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Studies on the Gamma Ray Irradiation on Protein

I. Effect on the Sulfhydryl Groups, Polarographic Patterns and Electron Spin Resonance Absorption of Bovine Serum Albumin

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(Received for publication, May 21, 1960)

Many investigations on the effect of radiation on living material have been published hitherto (*1*). Recently, Pollard (*2*) published a view that ionizing radiation would provide an efficient method for the studies of cellular function or structure, if its radical producing effect on the three key compounds of the cellular system, proteins, nucleic acids and polysaccharides, were made clear, because of the powerful, localised and penetrating nature of its action. With the progress in radiobiology and radiation medicine, investigations on its effects on these key compounds are increasing (*3*) and the effects have been chiefly attributed to the indirect effect of $OH^{\cdot}$, $H^{\cdot}$ or $HO_2^{\cdot}$ produced by irradiation of H_2O in the media (*4*). But, the radiation effect should be not only indirect but also direct. Therefore, the action of Co^{60} gamma rays on protein in solution has been compared with that in the solid state, using argentimetric titration and polarography to examine changes in free -SH groups of protein and in the pattern of the protein double waves in polarogram. Further, preliminary observations on the paramagnetic resonance absorption (ESR) of irradiated crystalline protein were also made.

MATERIALS AND METHODS

Irradiation of Protein—Armor's bovine serum albumin was used throughout the experiment. Protein dissolved in redistilled water (0.4 per cent) or in dried crystalline state was sealed in a Pyrex glass tube of Thunberg's type in the presence or absence (evacuated to 10^{-3} mm Hg) of air and exposed to gamma ray radiations of various doses. Solid samples were then dissolved in redistilled water (0.4 per cent) and subjected to the analyses. The irradiation was made by a Co^{60} gamma ray source having the intensity of 3×10^3 r per minute at the Institute for Chemical Research in Kyoto University. Irradiation was made at 8°C, and irradiated samples were stored in a refrigerator.

Argentimetric Titration—In the previous experiment (*5*) ammoniacal ethanol supporting media (pH 9.0) were used. But the occurrence of denaturation of protein and oxidation of -SH groups in such alkaline media in the presence of ethanol were highly probable (*6*), so that Tris-HNO_3 buffer (pH 7.4) was used.

Tris-HNO_3 solution (pH 7.4) was prepared by mixing 40 ml. of 1.0 *M* Tris, 34 ml. of 1.0 *M* HNO_3 and 3 ml. of 1.0 *M* KCl, and 12.8 ml. of this solution was diluted with redistilled water to 48 ml. before adding 2 ml. of 0.4% protein solution to be analysed. The protein-buffer mixture was titrated with 10^{-3} *M* $AgNO_3$ solution. As the electrodes, a Hg-HgO saturated Ba $(OH)_2$ half cell having the potential of −0.10 volt (*vsc*) and a Pt electrode rotating at 600 cps were used. The diffusion current of the Ag complex formed was read by a galvanometer 5 minutes after each addition of 0.01 ml. $AgNO_3$ solution.

Polarography—Using a Shimazu polarograph, observations were made on protein double waves in a Cobaltimmine-ammonium Buffer (pH 8.5) at 15°C, its composition being as follows: $Co(NH_3)_6$ Cl 0.001 *N*, NH_4Cl 0.1 *N*, NH_3 0.08 *N*. Protein concentrations were 4, 8, 16 and 24×10^{-4} per cent.

Paramagnetic Resonance Absorption—Observation of ESR absorption was made on solid protein samples by a hand-made spectrograph at the Institute of Physics, Kyoto University, its main part being constituted of a X band klystron operating at the constant frequency of 9400 *MC* per second and an electromagnet, of a pole with diameter being of 5 inches.

An adequate amount of solid samples was placed in a cell (6 mm × 10 mm × 2 mm) made of 0.3 mm.

Teflon plate and mounted in a H_{102} rectangular cavity situated between the poles of the electromagnet supplying a strong uniform magnetic field, which was modulated at 30 *cps* by means of two small coils mounted on the pole face of the magnet. The trace on the recorder chart was the first derivative of the absorption signal.

Stability and homogeneity of the magnetic field were sufficient to detect 10^{15} unpaired spins per ml. in the solid state. Indeed, some organic free radicals such as irradiated PMMA or Teflon were detectable, but the sensitivity or stability of the electromagnet or spectrometer was not sufficient for analysis of the hyperfine structure of irradiated protein. Therefore, the present observations amounted to only a detection of the presence of free radicals in the protein samples.

RESULTS

Argentimetric Titration—In the native bovine serum albumin, 0.67 titrable -SH groups per molecule were found. This value is compatible with that reported by Benesch *et al.* (*7*). Changes in the content of -SH groups caused by gamma ray irradiation (total doses 5×10^2 r, 10^3 r, 10^4 r and 10^6 r) under various conditions are presented in Table I; the albumin samples irradiated in the dissolved state were titrated 4 days after irradiation, while those in the solid state were dissolved in water two days after irradiation, stocked in a refrigerator and titrated 4 days after irradiation. A few observations carried out 1 or 2 hours after the irradiation gave similar results, with differences only in the absolute amount of change in the content of -SH groups.

As shown in this table, the effect of the presence of air (probably of oxygen) is very pronounced; amounts of titrable -SH groups are reduced in dissolved samples, and the oxygen effect is especially marked for small doses of gamma rays. Such a phanomendn would be attributable to oxidation of -SH groups as many workers have hitherto pointed out (*1*, *3*, *4*, *8*, *9*). On the other hand, little reduction of -SH group content was observed with the irradiated solid samples. Heavy irradiation (10^4 r or more) had, rather, an action of increasing their titrable -SH groups, suggesting that heavy irradiation produces configurational changes of protein molecules, for this

TABLE I

Change in the Content of Titrable -SH Groups of Irradiated Bovine Serum Albumin

Dose of γ-rays	No. of observation	Aq. solution in the presence of air	Aq. solution in the absence of air
		moles -SH/mole	moles -SH/mole
0	5	0.67±0.04	0.67±0.04
5×10^2	3	0.19±0.06	0.55±0.06
10^3	3	0.19±0.06	0.50±0.06
10^4	3	0.28±0.06	0.30±0.06
10^6	2	0.87±0.06	—[1]
		Solid state in the air	Solid state *in vacuo*
		moles -SH/mole	moles -SH/mole
5×10^2	2	—[1]	0.67±0.04
10^3	2	0.67±0.07	0.64±0.04
10^4	2	1.1 ±0.2	0.64±0.04
10^6	2	—[2]	0.97±0.04

1) Not measured.

2) Determination was impossible, because both samples were insoluble after irradiation.

dose is comparable with that for deaminating the protein (*9*). In the dissolved state, such a configurational change seemed to occur only at the high dose level of 10^6 r. In the solid samples, the effect of oxygen was also very evident; presence of air increased titrable -SH groups for irradiation of 10^4 r and heavier irradiation such as 10^6 r rendered protein insoluble. Little change in the -SH content is observed on samples irradiated *in vacuo*, and the large dose of 10^6 r produced only a moderate increase.

Paramagnetic Resonance Absorption—Gordy and his coworkers (*10*) investigated the ESR signals of X-ray irradiated protein and proved the existence of free radicals. In the present work some observations on the ESR signals of gamma ray irradiated crystalline bovine serum albumin have been made. Since the spectrometer and magnet used were not sensitive enough to detect lower densities of unpaired electrons with high reliability, a high dose such as 10^6 r was used. However, ESR signals seemed to appear in the samples ir-

radiated by gamma rays of 10^4 r. Observing their ESR signal at a typical X-band, the stable free radicals having gyromagnetic ratio (g) of about 2.0 could be detected for samples irradiated in the presence or absence of air. Even after keeping the sealed samples irradiated in the absence of air at ambient air temperature for 3 weeks, the presence of free radicals could be demonstrated, but signals could hardly be detected after such a sample was exposed to air for 5 days. We also failed to detect distinct signals, when protein samples irradiated in the presence of air (*i.e.* not evacuated) were examined one or more weeks after irradiation. These results seem to indicate that free radicals produced by irradiation are destroyed by reacting with oxygen for 1~3 weeks.

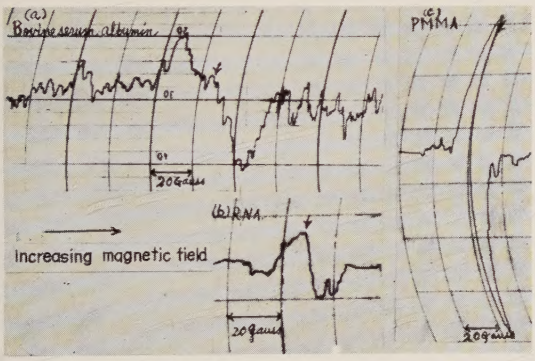

Fig. 1. Electron paramagnetic resonance absorption spectrum.

a: irradiated bovine serum albumin a few minute after exposure to air.

b: irradiated yeast RNA a few hours after exposure to air.

c: irradiated PMMA.

Arrow pointing downward represents the position for g=2,003. Each samples was irradiated with Co^{60} gamma ray (10^6 r) *in vacuo*.

As shown in Fig. 1, the signal of protein has a spacing far wider than that of PMMA or Teflon. Purified Yeast RNA (preparation of Takeda's Laboratory) irradiated under the same condition as albumin also gave ESR absorption having g=2.0, and its spacing (about 40 gauss) was narrower than that of albumin or PMMA. Such a narrowing would be attributable to the presence of an unpaired electron at the aromatic ring carbon and mainly originate from the exchange interaction (*11*). These results seem to suggest that unpaired electrons in protein free radicals would be of more than one kind, probably -S·, -C·, -O·, -O-O·, -S⋯S-, *etc.*, and other factors (*i.e.* inhomogeneous broadening) would also contribute to the broadening and asymmetry (*12*). Concerning the correlation between ESR absorption of irradiated albumin or RNA and doses of gamma rays, effect of the presence of oxygen or other factors, some observations are now being made with improved stability and sensitivity of our spectrometer. At present it might be said, that free radical formation occurs in the gamma ray irradiated protein and RNA, the mechanism probably being more complex in the former than in the latter.

Polarography—The polarograms of the native bovine serum albumin recorded were composed of two waves as already well known. The wave height was nearly proportional to the protein concentration within the range of 10^{-3}~10^{-4} per cent, with the second wave higher than the first one unless the concentration was less than 4×10^{-4} per cent. Irradiation caused a distinct change in the polarogram of protein; some examples of such effects of ir-

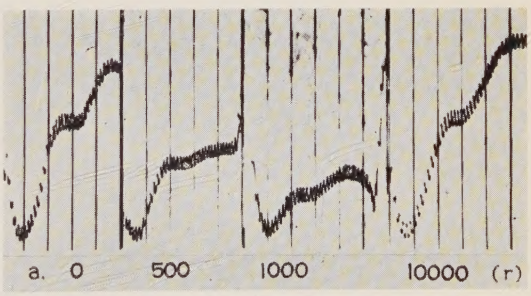

Fig. 2. Examples of polarograms of irradiated serum albumin.

The polarograms of the protein variously irradiated in the presence of air. Observation was made at the concentration of 2.4×10^{-3} per cent.

radiation are illustrated in Fig. 2. The samples were stocked in the refrigerator after irradiation, and the unirradiated control showed no observable change for a month when

stocked in the refrigerator.

As illustrated, polarograms of protein irradiated in the dissolved state show, the presence of oxygen, decreases in height of two waves for lower doses of gamma rays (5×10^2 r and 10^3 r), while wave patterns for heavy dose (10^4 r) papear, at a first glance, to show a tendency of recovering those height.

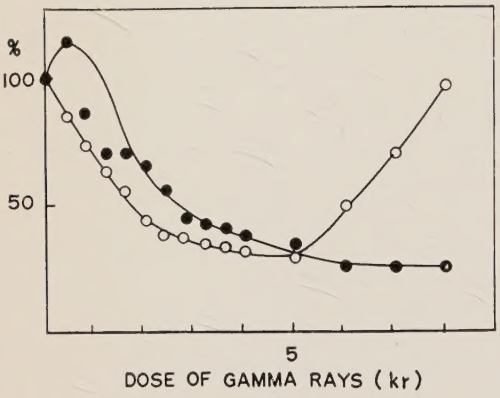

FIG. 3. Effect of gamma ray irradiation on the polarographic wave height of dissolved bovine serum albumin.

Absissa : dose of gamma rays in kilo-roentogens.

Ordinate : Per cent changes of the height of the first wave. —●— Immediately after, and —○— one day after irradiation.

These observations were made one day after irradiation. In one observation, therefore, the wave patterns recorded a few hours after the irradiation were compared with those after 24 hours or more. It was found that the heavier the irradiation the more the decrease in wave height, when observation was made a few hours after irradiation, as shown in Fig. 3. In this case, the content of titrable -SH groups also decreases even for high doses of irradiation of $10^3\sim10^4$ r, as constrasted to the values presented in Table I which were obtained a few days after irradiation. It might be said from such results, that increase in wave height of heavier irradiated protein does not run closely parallel with the change in titrable -SH groups.

Protein samples irradiated in the solid state gave enormously high waves and their patterns could not be recorded, when examined immediately after dissolution. After 30 minutes, however, protein wave patterns similar to, but somewhat higher than, those of an unirradiated control were observed even for such a heavy dose of gamma rays as 10^6 r. Those obtained after one day were usually stable, and a slight decrease in heights of the two waves were observed when irradiation was heavy. The change in wave height as time elapsed is illustrated in Fig. 4. In this

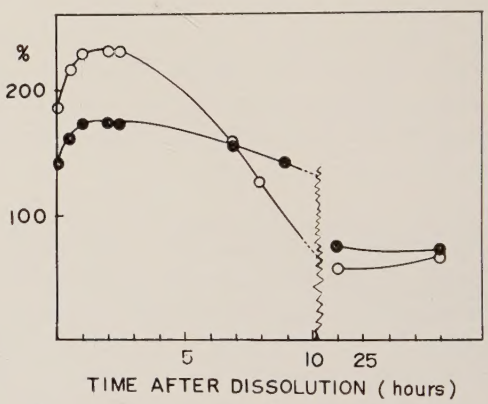

FIG. 4. Time course of polarographic change of serum albumin produced by heavy irradiation (10^6 r) in the solid state.

Absissa : time in hours after dissolution.

Ordinate : Per cent changes in the height of the first (●) and the second (○) waves.

case also, changes in wave height did not always run parallel with those in amount of titrable -SH groups.

DISCUSSION

Gordy and his co-workers (*13*) have already demonstrated in their ESR absorption studies that free radicals were produced by X-ray irradiation of amino acids and proteins or by gamma ray irradiation of hormones, vitamins and polysaccharides. In the present work, free radical formation in albumin molecule by gamma ray irradiation in the solid state was also proved. The dose of gamma ray to obtain a distinct ESR signal was 10^6 r or more, only a trace of free radicals being formed by a low dose such as 10^4 r.

The data in Table I, suggests the existance of a parallel between the change in the content of titrable -SH group and free radical formation in the irradiated albumin molecule. Moreover, its ESR signal has wide spacing and considerable asymmetry, showing considerable similarity to that of cystein or cystin (Gordy *et al.* (*14*); Imai, to be published). These facts appear to indicate that sulfhydryl or disulfide groups in albumin are considerably, though not entirely as postulated by Gordy *et al.*, for keratin concerned with radiation-induced changes, large part of free radical electrons being trapped on S atom.

Such free radicals in albumin were found stable *in vacuo* for a week. The fact that a very unstable, enormously high polarographic wave was observed only immediately after dissolution of such samples seems, therefore, attributable to some reaction of dissolved free radicals and these reaction would be also involved in determining their altered final polarographic wave patterns.

In the presence of O_2, as already well-known (*16*), radiation-induced free radicals would react with O_2, forming chiefly peroxide radicals. The restoration to the original form by recombination of free radicals is no longer possible but rather further, alteration of protein molecules should occur. Disappearance of the ESR signal of an irradiated sample after exposure to air for a week or more, as well as the fact that protein irradiated in the presence of O_2 increases its -SH group contents or becomes insoluble with a lower dose of gamma ray than that *in vacuo*, could be explained from such a point of view.

On the other hand, a view that the -SH group is very susceptible to radiation has been widely accepted (*15*). Indeed, in the present work a decrease in the amount of free -SH groups has been shown for protein irradiated with low doses of gamma ray ($5\times10^2\sim10^4$ r) in dissolved state, especially in the presence of O_2. For such low doses of radiation, however hardly any free radical formation was detected with the protein irradiated in the solid state. Accordingly, it would be attributable to oxidation of -SH group by free radicals produced from irradiated water molecules, primary effect of radiation on the protein molecules being their free radical formation and hence rather increase in the titrable -SH group content by denaturing process due to many sorts of free radical reaction.

Concerning the mechanism of the protein double waves, opinions have been divided, though there is no doubt that -SH and -S-S- groups in the molecule play an important role in determining the wave height (*17*, *18*). Systematic studies on the effect of high energy radiation on the protein wave have not yet appeared. It seems difficult, therefore, to explain the above-stated radiation-induced change of protein wave uniquely. It is very likely, that the observed changes are chiefly determined by the following factors: 1. Oxidation of the -SH group: 2. Unmasking of -SH or -S-S- groups produced by configurational changes of protein molecules and probably by the configurational changes themselves. The first one is mainly responsible for the reduction of wave height by lower dose irradiation, of the dissolved state, while augmented patterns obtained by high dose irradiation of the dissolved state as well as of the solid state result from the second. It seems worthy of note here that a heavily irradiated solution gave a low wave pattern for a few hour after irradiation, but its wave height increased after 20 hours or more, while reduction of its -SH content continued throughout the period of observation. A tentative explanation would be as follows: The free -SH group is very susceptible to rapid oxidation by irradiation in the dissolved state, low dose of radiation causes reduction of the protein wave height while heavier radiation causes results in the same effect at an earlier time. Configurational changes which occur at the same time under the latter condition proceed relatively slowly, so that the protein wave is augmented 20 hours or more after irradiation.

SUMMARY

1. Using a Co^{60} gamma ray source (intensity 3×10^3 r per hours), the effects of irradiation on bovine serum albumin in dissolved or

solid state were studied with amperometric titration of -SH groups, polarographic protein wave recording and paramagnetic resonance absorption spectroscopy.

2. The content of -SH groups was decreased when a protein solution was irradiated, especially in the presence of air, while hardly any reduction was observed on the solid samples in the absence of air. Such a decrease of -SH groups would result from oxidation. Increase in the amount of -SH groups observed on the solid samples irradiated in the presence of air could be attributable to the secondary configurational changes of molecules.

3. Marked changes of polarographic patterns of protein double waves were observed on both dissolved and solid samples and their time course or correlation with dose of radiation was studied.

These changes in protein waves did not always run parallel with those in the content of titrable -SH groups.

4. Formation of free radicals in the protein molecules by gamma ray irradiation could be proved with ESR absorption studies.

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Dephosphorylative Decomposition of Phosphagen (Phosphocreatine) by Zirconyl Ion*

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(Received for publication, July 18, 1960)

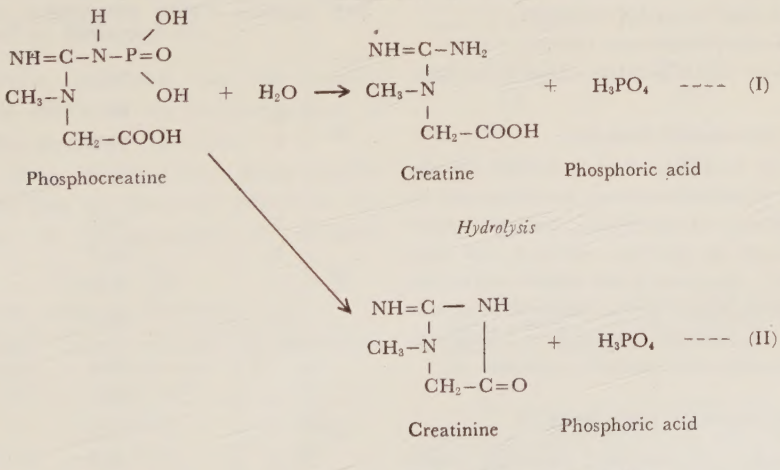
Phosphagen, *i.e.* phosphocreatine, is hydrolyzed by the action of hydrogen ion to its two components by the scission of its acid amide linkage. At the same time, another reaction occurs, as clarified by Iwasaki and Waseda (*1*), in which creatinine is formed as indicated in Scheme I. Based on experimental evidences, this reaction was considered to be one step reaction, in which the liberation of phosphoric acid from the phosphagen molecule and cyclization of creatine occurs simultaneously. This is therefore called dephosphorylative decomposition reaction. This dephosphorylative decomposition reaction of Iwasaki and Waseda occurs quantitatively in the presence of molybdenum. Assuming that there might be other substances which might effect such dephosphorylative decomposition, experiments were carried out with zirconium and this substance was found to have the action of catalyzing such decomposition reaction. Moreover, this substance was able to effect quantitative decomposition of phosphagen and this was proved by the result of Folin colorimetry (*2*) of creatinine thereby formed. At the same time, the amount of creatine formed was followed by Iwasaki's azotometry. The mode of this reaction was clarified by the method entirely different in principle from the Folin method.

MATERIALS AND METHODS

Experimental Materials

Phosphagen—Muscle was taken out from a frog (*Rana nigromaculata*) under ice-cooling and weighed. The muscle was ground, deproteinized by treatment with trichloroacetic acid, and the filtrate was neutralized to litmus with sodium hydroxide. This was

Scheme I



Dephosphorylative Decomposition

* The gist of this work was presented at the 69th Kanto Meeting of the Biochemical Society of Japan, November 8, 1952.

diluted with water so as to contain phosphagen in about 100 μg./ml. calculated as creatinine and the solution was stored in an ice chamber. Variation in the amount of phosphagen was examined by the Miyamoto's method (*3*) during storage and the values found are indicated in Table I. It was thereby found that there was almost no variation in the amount of phosphagen content over a fairly long period storage of this solution in an ice chamber.

TABLE I

Storage in ice chamber (days)	Amt. of creatinine from phosphagen in muscle-deproteinization soln. (μg./ml.)	Total creatinine in 0.1 g. of muscle (μg.)	Creatinine from phosphagen in 0.1 g. of muscle (μg.)	Percentage to total creatinine (%)
0	97.0	352	130.0	36.9
7	94.2		126.2	35.9
23	97.0		130.0	36.9
	mean 96.1			

	Creatinine already present in muscle-deproteinization soln. (μg./ml.)	Creatinine already present in 0.1 g. of muscle (μg.)	Percentage to creatinine from phosphagen (%)
25	2.0	2.7	2.1

Amt. of creatinine from phosphagen:
Deproteinized filtrate of muscle 1.0 ml., 10 g./dl. Sodium molybdate 0.5 ml., *N* HCl 1.0 ml.; Heated at 30°C for 10 min., measured by Folin's method.
Total creatinine:
Filtrate 1.0 ml., *N* HCl 1.0 ml.; Heated at 100°C for 90 min., measured by Folin's method.
Creatinine already present:
Measured directly by Folin's method.

Creatinine—Merck product was used.

Zirconyl Chloride—$ZrOCl_2 \cdot 8H_2O$ (Merck product) was used.

Experimental Methods

Determination of Creatinine—Folin method (*2*) was used. Standard creatinine solution was prepared so as to contain 100 μg. of creatinine, and the whole volume was brought to 12.5 ml., using a test tube graded to 12.5 ml. Reagents to be added to the test solution were always added to the standard solution.

Determination of Creatine—Iwasaki's azotometry by the sodium hypobromite method was used.

EXPERIMENTAL AND RESULTS

The quantity of zirconyl chloride sufficient to cause quantitative decomposition of phosphagen was examined and it was found that 0.5-1.0 ml. of aqueous solution containing 10 g./dl. (=0.31 *M*) of zirconyl chloride would be sufficient for 1 ml. of the test solution (containing phosphagen corresponding to *ca.* 100 μg. as creatinine). Therefore, 1.0 ml. of such the solution was used for subsequent experiments.

TABLE II

Effect of Heating

Temp. (°C)	Time (min.)	Amt. of creatinine formed from phosphagen (μg./ml.)	Decomposition rate (%)
30	3	76.7	79.9
	5	88.1	91.7
	7	91.5	95.2
	10	92.4	96.2
50	3	84.2	87.6
	5	95.2	99.0
	7	97.0	100.8
	10	96.0	99.9
70	1.5	96.1	100.0
	3	96.1	100.0
	5	97.0	100.8
	7	98.0	102.0
	10	97.0	100.8

Composition of the test solution:
Deproteinized filtrate of muscle 1.0 ml.
10 g. /dl. $ZrOCl_2$ 1.0 ml.
N HCl 1.0 ml.

Effect of Heating—As shown in Table II, experiments were conducted at 30°C, 50°C, and 70°C.

The above table shows that the decomposition reaches the maximum in 7 minutes at 30°C, 5 minutes at 50°C, and within 1.5 minutes at 70°C. It was thereby found that heating at 50°C for 6 minutes would be sufficient to effect complete decomposition.

Effect of Acidity—Examinations were made on the effect of acidity when decomposition of phosphagen is carried out with addition of zirconyl chloride. Hydrochloric acid was chosen as the strong acid and acetic acid as the weak acid, and experiments were carried out at *N*, *N*/10 and *N*/100 concentrations (Table III).

TABLE III

Effect of Acidity

Kind of acid	Acid concn. (*N*)	Amt. of creatinine formed (μg./ml.)	Decomposition rate (%)
HCl	1	95.2	99.0
	1/10	94.2	98.0
	1/100	93.3	97.1
CH_3COOH	1	95.2	99.0
	1/10	95.2	99.0
	1/100	92.4	96.1

Filtrate 1.0 ml., 1.0 g./dl. $ZrOCl_2$ 1.0 ml., acid 1.0 ml. Heated at 50°C for 6 min.

These results indicated that the acidity had almost no effect on the decomposition of phosphagen by zirconyl chloride.

Inhibitive Action of Phosphoric Acid—Acidity of the solution has no marked effect on the decomposition of phosphagen by zirconyl chloride but the use of phosphoric acid as the source of acidity has inhibitive action on zirconyl chloride (Table IV). At a concentration of *M* or *M*/10 phosphoric acid, there is a distinct inhibition of the decomposition reaction but a smaller amount, such as *M*/100, has no effect and the acid acts merely as same as hydrochloric acid. It is assumed that the inhibitive action of phosphoric acid is due to its combination with zirconyl chloride.

TABLE IV

Inhibitive Action of Phosphoric Acid

Kind of acid	Acid concn. (*M*)	Amt. of creatinine formed (μg./ml.)	Decomposition rate (%)
H_3PO_4	1	56.5	69.2
	1/10	76.8	80.7
	1/100	93.2	97.0
HCl	*N*/10	93.2	97.0

Conditions of test were the same as shown in Table III.

TABLE V

Inhibitive Action of Phosphate

Amt. of KH_2PO_4 (g./dl.)	Amt. of KH_2PO_4 (ml.)	Amt. of creatinine formed (μg./ml.)	Decomposition rate (%)
10	1.0	78.7	68.1
,,	0.5	83.3	72.5
1	1.0	89.3	77.6
,,	0.5	105.3	91.5
,,	0.4	108.7	94.5
,,	0.3	115.0	100.0
,,	0.2	115.0	100.0

Phosphagen solution (115.0 μg./ml. as creatinine) 1.0 ml., 10 g./dl. $ZrOCl_2$ 1.0 ml., *N* HCl 1.0 ml., KH_2PO_4 as listed in the table, water to bring to a final volume of 4.0 ml. Heated at 50°C for 6 min.

TABLE VI

Effect of $ZrOCl_2$ on Phosphate Inhibition

Amt. of 10 g./dl. $ZrOCl_2$ added (ml.)	Amt. of creatinine formed (μg./ml.)	Decomposition rate (%)
1.0	89.3	77.6
1.2	95.3	82.9
1.3	100.0	86.9
1.35	107.6	93.5
1.4	115.0	100.0
1.5	117.6	102.4

Phosphagen solution (115.0 μg./ml. as creatinine) 1.0 ml., 1 g./dl. KH_2PO_4 1.0 ml., 10 g./dl. $ZrOCl_2$ as listed, *N* HCl 1.0 ml. Heated at 50°C for 6 min.

In order to examine this point further, decomposition reaction was carried out with a crude sample of phosphagen added with potassium dihydrogenphosphate and, as shown in Table V, there was a distinct inhibition. This inhibition was found to disappear by the increased quantity of zirconyl chloride (Table

VI). From these tables, it is seen that 1 mole of potassium dihydrogenphosphate corresponds to *ca.* 2.6 moles of zirconyl chloride.

Inhibitive Action of Sulfate—In order to see the effect of sulfate, experiments were then carried out with potassium sulfate and, as shown in Table VII, there was a distinct inhibitive action but this was also found to disappear on increasing the amount of zirconyl reagent (Table VIII). From these tables, it is seen that 1 mole of potassium sulfate corresponds to *ca.* 1 mole of zirconyl chloride.

TABLE VII

Inhibitive Action of Sulfate

Amt. of K_2SO_4 (g./dl.)	Amt. of K_2SO_4 (ml.)	Amt. of creatinine formed (μg./ml.)	Decomposition rate (%)
5	1.0	41.4	36.0
,,	0.5	85.4	74.2
,,	0.4	91.6	79.7
2	0.8	104.2	90.6
,,	0.7	108.7	94.4
,,	0.6	115.0	100.0
5	0.2	116.3	101.1
0.5	1.0	117.6	102.4
,,	0.5	117.6	102.4

Phosphagen solution (115.0 μg./ml. as creatinine) 1.0 ml., 10 g./dl. $ZrOCl_2$ 1.0 ml., *N* HCl 1.0 ml., K_2SO_4 as listed, water to bring to a final volume of 4.0 ml. Heated at 50°C for 6 min.

TABLE VIII

Effect of $ZrOCl_2$ on Sulfate Inhibition

Amt. of 10 g./dl. $ZrOCl_2$ added (%)	Amt. of creatinine formed (μg./ml.)	Decomposition rate (%)
1.00	91.6	79.7
1.05	100.0	86.9
1.10	103.1	89.7
1.15	115.0	100.0
1.20	116.3	101.1

Phosphagen solution (115.0 μg./ml. as creatinine) 1.0 ml., 2 g./dl. K_2SO_4 1.0 ml., 10 g./dl. $ZrOCl_2$ as listed, *N* HCl 1.0 ml. Heated at 50°C for 6 min.

Decomposition with Acid alone, without Addition of Zirconyl Chloride—As shown in Table IX, decomposition reaction hardly takes place in the absence of zirconyl chloride but this does not mean that the remainder has wholly undergone hydrolysis, because it is clear from the fact that the amount of detectable creatinine increases by subsequent addition of zirconyl chloride and heating at 50°C for 6 minutes after treatment with acid alone at 50°C for 6 minutes.

Foregoing experiments have clarified fundamental conditions necessary to effect complete decomposition of phosphagen in the presence of zirconyl chloride.

Comparison of Molybdenum and Zirconium Methods—The behavior of phosphocreatine and its components and derivatives to various

TABLE IX

Decomposition with Acid Alone

Kind of acid	Acid concn.	Amt. of creatinine formed (μg./ml.)	Decompn. rate (%)	Creatinine formed from rest of phosphagen by addn. of $ZrOCl_2$ (μg./ml.)	Decompn. rate (%)
HCl	*N*	7.1	7.4	39.7	41.3
	N/10	4.2	4.4		
	N/100	2.9	3.0		
H_3PO_4	*M*	6.6	6.9	28.5	29.7
	M/10	3.6	3.7		
	M/100	4.1	4.3		

Deproteinized filtrate of muscle 1.0 ml., Acid 1.0 ml. Heated at 50°C for 6 min.
10 g./dl. $ZrOCl_2$ solution 1.0 ml. added and heated at 50°C for 6 min.

methods is listed in Table X.

It is therefore seen that if the reaction is started by giving some chemical stimulation on phosphocreatine under various conditions, it should be decomposed into some of the substances listed in Table X and these decomposition products could be determined by the respective method of determination. This is illustrated in Fig. 1. Based on the facts revealed by the study of Iwasaki's azotometry, comparative experiments were carried out with the addition of molybdenum or zirconium reagent.

TABLE X

Substance	Hypobromite azotometry	Folin's method	Fiske-Subbarow's method (4)
Phosphocreatine	−	−	−
Creatine	+	−	−
Creatinine	−	+	−
Phosphoric acid	−	−	+

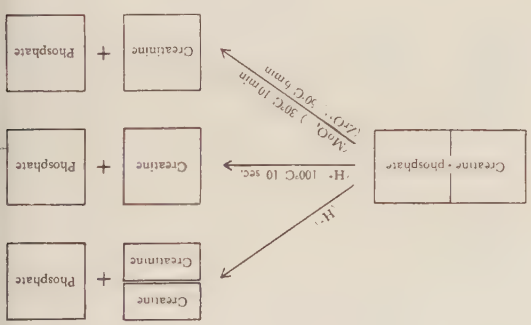


FIG. 1.

Determination of Phosphagen by Iwasaki's Azotometry—For the quantitative hydrolysis of phosphagen, hydrolysis tube devised by Prof. K. Iwasaki was utilized. In this tube, 1.0 ml. of the deproteinized filtrate of frog muscle was placed, 1.0 ml. *N* hydrochloric acid was added, and the mixture was heated at 100°C for 25 seconds. This solution was neutralized with 0.1 ml. of 10 *N* sodium hydroxide and the mixture was submitted to Iwasaki's azotometry. In this case, a part of free creatine present in the muscle and creatine formed by hydrolysis of phosphagen would form creatinine, though in extremely small amount. The creatinine thereby formed was therefore measured by Folin's colorimetry and the value was added to the amout found by the azotometry. The value obtained by subtracting the blank value from this sum would be the amount of creatine formed from phosphagen. Blank azotometry values were obtained by the molybdenum or zirconium method, by which quantitative decomposition of phosphagen was effected by the us eof molybdenum or zirconium, whereby the componental creatine would be converted to creatinine at the same time, and the solution so obtained was submitted to the azotometry.

Determination of Phosphagen by Colorimetry—Quantitative decomposition of phosphagen was carried out with the use of molybdenum or zirconium reagent and the amount of creatinine was determined by Folin's colorimetry.

As shown in Table XI, the values in azotometry by the molybdenum and zirconium methods were in good agreement. The values in colorimetry by two methods also showed good agreement. Moreover, the value of phosphagen by the azotometry and colorimetry were also in good agreement.

The deproteinized filtrate of frog muscle contains, besides creatine produced from phosphagen, free creatine, urea, and ammonia, which produce nitrogen by the hypobromite azotometry and the blank value in azotometry tends to become greater (column b in Table XI). This in turn tends to increase the error in this azotometry. In order to remove these factors, phosphagen was purified by the Fiske-Subbarow method (5) and approximately pure sample was obtained. A definite quantity of this purified sample was dissolved in water and the same experiments were carried out. Results thereby obtained are listed in Table XII in which the blank value for nitrogen (column b) is only very minute from the absence of nitrogenous substances like free creatine, urea, and ammonia.

TABLE XI

Comparison of Molybdenum and Zirconium Method with Deproteinized Filtrate from Frog Muscle

Expt. No.	Method	Azotometry						Colorimetry			
		N_2 after acid hydrolysis (μl.) (a)	N_2 from blind test (μl.) (b)	N from creatinine from acid hydrolysis[1] (μl.) (c)	Phosphagen		Calcd. with Mo method value as 100 (%)	N from total creatinine[2] (μg.) (d)	N from creatinine in blank[3] (μg.) (e)	N from creatinine in phosphagen (μg.) (d)−(e)	Calcd. with Mo method value of azotometry as 100 (%)
					N_2 (μl.) (a)−(b)+(c)	Detected as N (μg.)					
I	Mo	147.8	94.0	3.0	56.8	106.5	100.0	108.6	1.9	106.7	100.2
	Zr		93.7		57.1	107.3	100.8	107.1		105.2	98.9
II	Mo	150.6	118.7	1.9	33.8	63.3	100.0	64.6	2.0	62.6	98.4
	Zr		120.4		32.1	60.2	95.1	64.6		62.6	98.4
III	Mo	136.4	103.4	2.7	35.7	66.9	100.0	70.4	1.6	68.8	102.7
	Zr		101.2		37.9	71.0	106.1	70.6		69.0	103.2

1) Measured by colorimetry and converted to N_2 from the azotometry.
2) Nitrogen from creatinine in blind value+nitrogen from phosphagen creatinine.
3) Naturally present creatinine.

TABLE XII

Comparison of Molybdenum and Zirconium Methods in Phosphagen Solution

Expt. No.	Method	Azotometry						Colorimetry			
		N_2 after acid hydrolysis (μl.) (a)	N_2 from blind test (μl.) (b)	N from creatinine from acid hydrolysis[1] (μl.) (c)	Phosphagen		Calcd. with Mo method value as 100 (%)	N from total creatinine[2] (μg.) (d)	N from creatinine in blank[3] (μg.) (e)	N from creatinine in phosphagen (μg.) (d)−(e)	Calcd. with Mo method value of azotometry as 100 (%)
					N_2 (μl.) (a)−(b)+(c)	Detected as N (μg.)					
I	Mo	38.9	0.2	2.4	41.1	77.0	100.0	75.6	0.3	75.3	97.8
	Zr		0.2		41.1	77.0	100.0	74.8		74.5	96.8
II	Mo	36.9	0.8	4.3	40.4	75.8	100.0	73.4	0.3	73.1	96.5
	Zr		0.6		40.6	76.1	100.4	73.5		73.2	96.6

1) 2) 3) } Same as Table XI

SUMMARY

Foregoing experiments have proved that zirconyl chloride has the ability to cause decomposition reaction when applied to phosphagen and fundamental conditions necessary to effect quantitative decomposition of phosphagen by the use of zirconium have been established.

In the determination of phosphagen by Iwasaki's azotometry, the values by the molybdenum or zirconium method were in good agreement, and well agreeing values were also obtained by Folin's colorimetry. It therefore follows that a quantitative decomposition of phosphagen has been effect-

ed by zirconyl chloride. The decomposition of phosphagen by the use of zirconium reagent is considered to be the dephosphorylation from the phosphagen molecule, as by the use of molybdenum reagent. It is also assumed that hafnium derivative, which has practically the same chemical properties as that of zirconium, would behave similarly towards decomposition of phosphagen.

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Studies on Histidine Residues in Hemeproteins Related to Their Activities

V. Photooxidation of Catalase in the Presence of Methylene Blue

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It has been reported in the papers of this series, that cytochrome c completely lost its enzymatic activity, when one mole of histidine residues was destroyed by photooxidation (*1*), while photooxidized peroxidase maintained its complete enzymatic activity, even when 85 per cent of the histidine residues was destroyed (*2*). In the present communication, the effect of photooxidation on catalase will be described in relation to the enzymatic activity and histidine residues in the enzyme.

EXPERIMENTAL

Preparation and Assay of Catalase—Catalase was prepared from a fresh bovine liver, by the method of Shirakawa (*3*), and was recrystallized several times before being used in the experiment. Catalase activity was determined by Euler and Josephson's method (*4*), and K_f value of the sample used was 32,000.

Preparation of Hemoglobin—Crystalline hemoglobin was prepared from bovine erythrocytes as described previously (*5*), and used for viscosity measurement after lyophilization. Lyophilized sample contained about 10 per cent moisture.

Photooxidation—All applied techniques except the measurement of viscosity have been described previously (*1*, *2*).

Measurement of Viscosity—In Ostwald viscosity meter, 2.5 ml. of catalase solution, 2 ml. of 0.2 *M* phosphate buffer of pH 7.4, and 0.5 ml. of 0.02 per cent methylene blue were added and the mixture was irradiated under similar conditions of light illumination to those in the photooxidation experiment. With hemoglobin, lyophilized sample in 2.5 ml. was used instead of catalase solution.

Determination of Amino Acids—The content of each amino acid in the reaction mixture during photooxidation was determined. Tryptophan was determined by the *p*-dimethylaminobenzaldehyde method (*6*), after removing the methylene blue with the addition of a small amount of charcoal. Histidine (*7*) and tyrosine (*8*) were determined by means of the same methods as described in a previous paper (*5*). The protein concentration, and N contents in a hydrolyzed solution were determined by micro-Kjeldahl method.

RESULTS

Oxygen Uptake during the Photooxidation of Catalase—Two ml. of catalase solution, 1.0 ml. of 0.1 *M* phosphate buffer, pH 7.4, and 0.3 ml. of 0.2 per cent of methylene blue were placed in a Warburg vessel and photooxidation was performed. The pH of the reaction mixture was adjusted to 7.4, in order to prevent the

Table I

Oxygen Uptake during the Photooxidation of Catalase

Irradiation Time (min.)	Catalase mg.[1]			
	9.2	4.6	2.3	2.3[2]
5	14.2[3] μl.	7.3	4.6	1.3
10	26.8	15.6	9.2	2.0
20	36.2	22.1	13.8	3.8
30	49.0	29.2	20.0	5.0
60	73.8	47.0	30.8	7.5
90	94.0	59.2	40.0	8.8
120	108.7	68.2	46.0	10.2
180	131.6	79.6	53.2	13.0

1) Catalase mg. in each vessel.
2) Absence of methylene blue.
3) O_2 μl. uptaken.

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inactivation of catalase by an alkaline pH (Table I).

In the absence of methylene blue, catalase was only slightly oxidized by irradiation, while in the presence of methylene blue, no oxygen was consumed in the absence of light radiation. Since the oxidation of protoporphyrin was negligible under the conditions used here, as reported previously (*1*), oxygen uptake observed was considered to be due primarily to the oxidation of the protein moiety of catalase by methylene blue sensitized photochemical action. A similar phenomenon was also observed in other proteins (*1*, *2*, *9*, *10*).

Decrease in Catalase Activity and Amino Acids Contents during Photooxidation—After certain periods of irradiation in Table II, 0.2 ml. of the reaction mixture was taken from a vessel and diluted 100 to 2,500 times with *M*/15 of phosphate buffer (pH 6.8), and at this proper concentration catalase activity was assayed. At the same time, amino acid content of each corresponding reaction mixture was assayed. Taking 1 ml. of reaction mixture, tryptophan was determined and the remaining reaction mixture was hydrolyzed to determine histidine and tyrosine. The analytical values of histidine, tyrosine and tryptophan contents of catalase obtained in control experiment were 6.0, 5.7 and 1.7 per cent respectively, based on N content of 16.8 per cent. The decrease in each amino acid during the photooxidation is shown in Table II.

Table II

Decrease in Various Amino Acids and Catalase Activity caused by the Irradiation

Irradiation Time (min.)	O_2 mole uptaken	Remaining His. %	Remaining Tyr. %	Remaining Try. %	Activity %
5	22.1	68.5	91.0	96.5	84.8
10	35.2	58.0	88.6	95.7	73.2
20	52.0	49.2	73.8	95.5	56.5
30	61.0	43.0	72.5	95.3	43.0
60	97.0	28.5	72.0	95.0	18.0
90	124.0	19.0	71.0	94.8	7.9
120	138.0	12.8	70.5	94.5	3.4

Photochemical Action on the Absorption Spectrum of Catalase—The spectra of photooxidized catalase were measured after treating the reaction mixture with a small amount of charcoal to remove methylene blue (Fig. 1).

The absorbance at the regions of 405 mμ and 500 to 600 mμ were remarkably changed by photooxidation. It was interesting to compare the decrease in the absorbancy at 405 mμ with that in catalase activity, since the absorbancy at this region was considered to be due, in part, to the heme-protein linkage as described in previous papers (*1*, *2*). Each

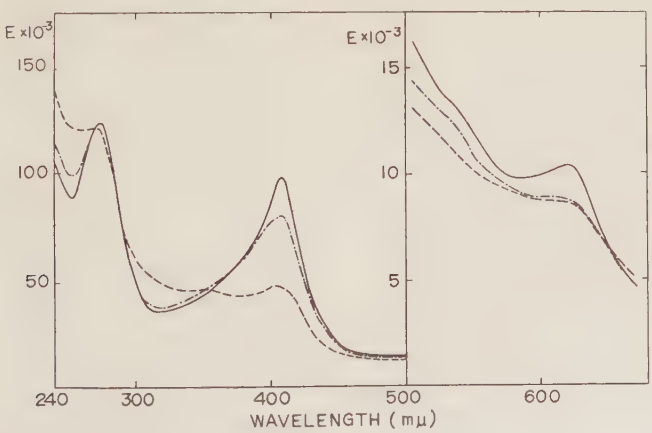

Fig. 1. Photochemical action on the absorption spectrum of catalase. Curve I ——: Native catalase, Curve II —·—: Photooxidized for 10 min., and Curve III – – –: Photooxidized for 120 min.

absorbancy was plotted against time, and the value after 150 minutes irradiation was graphically found to be a minimum one. Hence a long period of photooxidation caused catalase to bind with methylene blue and the binding substance could not be removed by charcoal. The value obtained graphically for 150 minutes of irradiation, corresponding 49 per cent of initial absorbancy, was used for the calculation of decreasing ratio of absorbancy. The ratio, $(D_t\text{-}D_{150})/(D_0\text{-}D_{150})$, was calculated ($D_0$, D_{150} and Dt represent per cent of absorbancy at 405 mμ at 0, 150 and t minutes of irradiation, respectively). A fairly agreeable relationship between catalase inactivation and decrease in absorbancy was shown in Table III.

TABLE III

Effect of Photooxidation on Catalase Activity and Absorbancy at 405 mμ

Irradiation Time (min.)	Remaining Activity %	O. D.$_{405}$%	$\frac{D_t-D_{150}}{D_0-D_{150}}\times 100$
5	84.8	93.6	87.0
10	73.2	88.3	76.4
20	56.5	80.8	61.2
30	43.0	75.0	49.6
60	18.0	62.1	23.4
90	7.9	56.0	11.1

D_0, D_{150} and D_t represent per cent of optical density at 405 mμ at the irradiation time of 0, 150 and t minutes.

Photochemical Effect on Viscosity of Catalase

Weil *et al.* have shown that no detectable change occurred in the solubility and viscosity of photooxidized chymotrypsin (*9*), which consumed 9.8 moles of oxygen per mole of the protein, and concluded that denaturation had not been caused by photooxidation of this enzyme.

The relative viscosity change of catalase and hemoglobin were determined during photooxidation, since a part of histidine residues were shown to be masked in native state (*5*) and some of them might be related to the secondary structure. With 14.5 mg. of catalase in 5 ml. of reaction mixture, the relative viscosity was measured at 0, 60 and 90 minutes of irradiation: t=0, ηr=1.006; t=60, ηr=1.008; and t=90, ηr=1.008. Using 50 mg. of lyophilized sample of hemoglobin, the viscosity change was also measured: t=0, ηr=1.032; t=60, ηr=1.033; and t=90, ηr=1.036.

From the viscosity data, it is considered that the shapes of the catalase and hemoglobin were modified to some extent by the photooxidation, though at the beginning o the irradiation the change of protein shape might not be enough to cause a viscosity change.

DISCUSSION

Catalase which was subjected to the photooxidation in the presence of methylene blue showed a decrease in enzymatic activity, and the rate of inactivation was proportional to the irradiation time. As shown in other hemeproteins (*1*, *2*), the oxygen uptake during photooxidation was considered to be due to the destruction of amino acids. Among these amino acids, histidine residues were mainly destroyed and other amino acids, tyrosine and tryptophan residues, were destroyed only to some extent. At the initial stage of photooxidation, destruction of histidine residues was rapid, but the rate of destruction did not parallel the inactivation of catalase. The total histidine destroyed was thus analyzed into two groups, "fast" and "slow" destructable groups, according to Koshland *et al.* (*11*). No direct correlation between the inactivation of catalase and the rate of both "slow" and "fast" phases of destruction existed. It was assumed that destruction at the later period of irradiation was due to the "slow" destructable histidine groups. By extrapolating a straight line of destruction of histidine in Fig. 2, this group was found to comprise 65 per cent of the total histidine. The "fast" group was calculated by subtracting the "slow" histidine from the total histidine destroyed, andw as found to be 35 per cent of the latter. As it has been calculated that one molecule of catalase contained approximately 95 moles of

histidine residues, 62 and 33 moles of histidine residues could be classified as the "slow" and "fast" groups respectively.

In contrast with the data of phosphoglucomutase (*11*) or cytochrome c (*1*), it appeared that histidine residues belonging to the "slow" destructive group were responsible for the enzymatic activity of catalase. The decrease in enzymatic activity, however, did not parallel the ratio of destruction of "slow" histidine, but seemed to have a square or higher power relation with the latter.

According to Koshland *et al.* (*11*), the following equations were respectively given for the cases; (i) two histidines were involved at the site of enzymatic activity and both were required for the activity, and (ii) either alone was able to act in the enzymatic action.

(i) $(A/Ao)=(H/Ho)^2$

$$\frac{d(A/Ao)}{dt}=2(H/Ho)\frac{d(H/Ho)}{dt} \qquad (1)$$

(ii) $(A/Ao)=-(H/Ho)^2+2(H/Ho)$

$$\frac{d(A/Ao)}{dt}=\left(-2H/Ho+2\right)\frac{d(H/Ho)}{dt} \qquad (2)$$

(A and Ao: enzymatic activity at t and 0 time, and H and Ho: histidine content at t and 0 time).

The relation between "slow" destructive histidines and decrease in enzymatic activity was calculated according to the above equations, and considerable agreement between "slow" histidine and inactivation was shown, using the above equation (*1*) (Table IV). These results of the kinetic analysis showed that two histidines were involved in the active site of enzyme, but either alone did not act in the enzymatic activity, since the relationship of the equation (*2*) was not upheld.

It was shown by Chance that 1.2 to 1.6 moles of H_2O_2 were involved in the complex I, an enzyme-substrate complex at the first stage of the enzyme action (*12*, *13*). It was interesting to compare the amount of H_2O_2 in complex I with the number of histidine residues required for enzymatic activity obtained by the above kinetical analysis, since the active site(s) in catalase might possibly have a function to bind H_2O_2.

In the previous paper (*1*), only one histidine was shown to be required for the activity of cytochrome c, in which one heme is attached. It might be possibly considered that one histidine is required for maintaining the enzymatic activity of one heme. The H_2O_2 which binds to catalase at the first stage of the complex might bind at two different active sites, where each of two histidine might be involved.

Though the results of relative viscosity suggested some change in protein structure, catalase activity could be maintained by a limited part of the protein, since only two

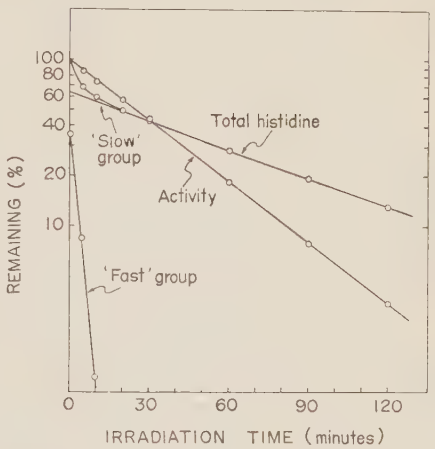

Fig. 2. Effect of irradiation time on histidine content and enzymatic activity of catalase.

Table IV

Relation of Catalase Activity and 'slow' Destructive Histidine Group

Irradiation Time (min.)	H/Ho	2H/Ho×ΔH/t	ΔA/t
5	.9320	.0284	.0304
10	.8646	.0181	.0236
20	.7569	.0164	.0167
30	.6615	.0123	.0135
60	.4384	.0067	.0083
90	.2923	.0028	.0037
120	.1969	.0012	.0015

Ratio of
H/Ho: remaining 'slow' histidine
ΔA and ΔH: Infinitestimal decreases in enzymatic activity and histidine respectively.

histidine residues were shown to be required for the enzymatic activity.

Destruction of total histidine was analyzed into 2 straight lines of "slow" and "fast" destructable groups, according to Koshland *et al.*

Absorption at 405 mμ decreased remarkably after the irradiation, and a considerable agreement between the rates of the decrease in absorption at this region and the inactivation of enzymatic activity was shown. As it was discussed in previous papers of this series, the absorption at this wave length could be explained to be due partly to the heme-protein linkage (*1*, *2*). With carboxymethylation of hemoglobin and catalase, remarkable decreases in the absorption at this region was observed in both cases (*5*, *14*) .In this reaction, carboxymethylation of histidine, tyrosine and lysine residues was known to occur in many proteins (*15*, *16*). With hemoglobin, formation of methemoglobin might have occurred and caused the decrease in the absorbancy. However, heme iron in catalase is in the ferric state, the decrease might be then possibly due to the modification of protein moiety.

Histidine specific photooxidation of cytochrome c also resulted in a decrease in the above absorption, whereas peroxidase did not change the absorption after photooxidation (*1*, *2*). Since cytochrome c has histidine-heme linkage, and peroxidase was proposed to involve no histidine-heme linkage, the results with catalase photooxidation appeared to show that there might be possibly histidine-heme linkage. If histidine residues are coordinated with the heme iron or situated closely enough to effect the heme-protein linkage, the break of this residues would result in the change in absorption of the above region. Thus, in catalase histidine residues are possibly proposed to be situated at the position to influence the heme-protein linkage.

SUMMARY

1. Catalase was photooxidized in the presence of methylene blue, resulting in a decrease in enzymatic activity and a destruction of amino acids. Following the kinetic analysis, the square of the remaining histidine residues was proportional to the remaining enzymatic activity during photooxidation. This indicates that two histidines are required for the enzymatic activity.

2. Absorption spectrum of catalase was changed by photooxidation and the absorbancy at 405 mμ was prominently decreased as with cytochrome c, but not as with peroxidase. The rate of decrease in absorption at 405 mμ and of inactivation of catalase during photooxidation were compared, and a close relationship was shown between these two rates.

The author wishes to express gratitude to Prof. Y. Oshima and Prof. M. Funatsu for their invaluable advice and criticism during this work.

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Biochemical Studies on Sulfate-Reducing Bacteria

IX. Sulfite Reductase

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Sulfite has been conceived to be an intermediate of sulfate reduction in sulfate-reducing bacteria, *Desulfovibrio*, as well as in other microorganisms and higher plants. Ishimoto (*1*) and Koyama *et al.* (*2*) observed a preferential utilization of sulfite to sulfate as a hydrogen acceptor when they were present together in suspension of *Desulfovibrio* and suggested the possibility of intermediary formation of sulfite in sulfate reduction. Millet demonstrated the formation of sulfite in the reduction of sulfate by means of radioisotope in another strain of the bacterium (*3*). Recently Ishimoto *et al.* (*4*) and Peck (*5*) found the intermediary formation of adenosine-5′-phosphosulfate and of sulfite in this order in the course of sulfate reduction in the *Desulfovibrio* extracts. The formed sulfite had been reported to be reduced to sulfide by the cell-free extracts (*6*, *3*). Postgate reported recently that in the reduction of sulfite in the extracts several protein fractions besides cytochrome c_3 and hydrogenase as well as non-proteinous factors were essential (*7*).

In yeast, sulfite is also an intermediate of sulfate reduction, but in this case the preceding substance is phosphoadenosine-5′-phosphosulfate (*8*, *9*). The sulfite formed is reduced to sulfide by the same extracts.

As the enzymic mechanism of the sulfite reduction has not yet been completely elucidated, the authors intended to devise an assay method for the sulfite reductase employing methylviologen as an electron carrier, to purify the enzyme of *Desulfovibrio*, and to study the properties of the reductase as well as the participation of cytochrome c_3 in the reduction. A part of the meterial of this paper has already been published briefly elsewhere (*10*, *11*).

METHODS AND MATERIALS

Preparation of Cell-Free Extract—The strain of sulfate-reducting bacterium and culture method were the same as those reported in an earlier paper (*12*). Cell-free extract was obtained by disintegration of the cells by a sonic or ultrasonic oscillator followed by centrifugation for 20 minutes at $6,000 \times g$ to eliminate cell debris.

Preparation of Hydrogenase—The crude extract was centrifuged for 20 minutes at $18,000 \times g$ and the precipitate obtained was, after washing, used as the preparation of hydrogenase. It contained scarcely any activity of the reductase (*13*).

Assay of Sulfite Reductase—Two methods were devised. One method was to measure manometrically the rate of consumption of hydrogen in the presence of hydrogenase, sodium sulfite and methylviologen as an intermediary electron carrier. Reaction mixture contained 75 μmoles of phosphate buffer, pH 6.2, 4 μmoles of sodium sulfite, 1 μmole of methylviologen, hydrogenase preparation (10 mg. protein) and enzyme preparation in a total volume of 1 ml. The atmosphere was hydrogen and temperature was 30°C. The reaction was started by dumping the solutions of sulfite and methylviologen from the side arms of the vessels. The rate of hydrogen uptake was determined by the linear part of the time course curve and the unit of the enzyme activity was defined as the amount of the enzyme which consumed 1 μmole of hydrogen in one hour. The velocity was proportional to the amounts of the enzyme in the range of 1–15 units.

The other method of assay was to measure the time of oxidation of reduced methylviologen by sulfite using Thunberg tubes. As methylviologen is blue in the reduced form and colorless in the oxidized,

the presence of the enzyme was indicated by the fade of the color and the reaction velocity was measured roughly by the decolorization time. Reaction mixture consisted of 4 μmoles of reduced methylviologen, 10 μmoles of sulfite, 100 μmoles of phosphate buffer, pH 6.0, and enzyme preparation in total volume of 5 ml. Carbon monoxide was charged over the reaction mixture with a pressure of 0.5 atmosphere to inhibit hydrogenase which caused the decolorization of reduced methylviologen with evolution of hydrogen in the absence of carbon monoxide. Time of decolorization was measured at 30°C. One unit of sulfite reductase was defined as the amount of enzyme which oxidized two micromoles of reduced methylviologen in one hour. When the partially purified preparation was employed, the decolorization time was inversely proportional to the added amounts of the preparation in the range of 1–10 units (Fig. 1).

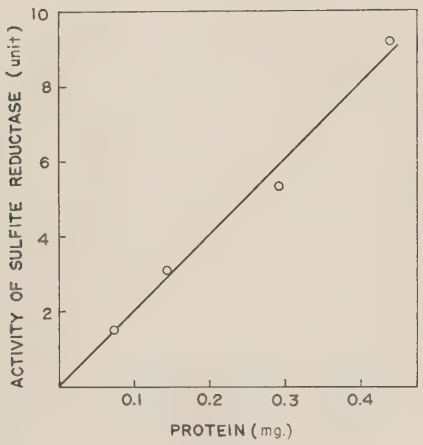

Fig. 1. Proportionality of activity and amounts of the sulfite reductase.

Assay of Other Enzymes—Assay of thiosulfate reductase had been reported in the previous paper (*13*) and that of hydrogenase was Tamiya's method (*14*).

Amounts of proteins in the extracts and preparations were determined by Johnson's bichromate method for the estimation of non-volatile organic matters (*15*).

Reagents—Methylviologen was a product of the British Drag Houses Co. Reduced methylviologen was prepared by warming methylviologen solution with zinc sand in dilute phosphate buffer under reduced hydrogen atmosphere (*16*).

RESULTS

Partial Purification of Sulfite Reductase—Suspension of cells of *Desulfovibrio* (33 g. in 50 ml. of water) was treated with ultrasonic oscillation (560 kc) for 10 minutes. The crude extract was centrifuged at 18,000$\times g$ for 20 minutes and the precipitate was suspended in water after washing. Saturated solution of ammonium sulfate was added to the supernatant solution and the precipitates between 0.33 and 0.67 saturation were dissolved in water and dialysed against water. To the supernatant of the dialyzed solution (50.5 ml.), calcium phosphate gel was added three times (each time 0.60 g. per 1 g. protein) and centrifuged down after each 15 minutes'

Fig. 2. Starch column zone-electrophoresis of crude extract.

Sonic extract submitted to ultracentrifugation at 100,000$\times g$ for one hour and passed through a column (1.5$\times$7.0$\times$40 cm.) of Amberlite IRC 50 (Na^+ type) to eliminate cytochrome c_3 and dialyzed against distilled water for 40 hours with change of water three times. The dialyzed preparation was subjected to zone electrophoresis on Starch base, which had been washed with 0.05 N NaOH, distilled water, and finally with 0.02 M KH_2PO_4 and adjusted to PH 7. 7 ml. of the dialyzed preparation (Protein 19 mg./ml.) was charged and electrophoresis was carried out at 300 volt, 0.025 A for 10 hours at 4°C.

PROT. Concentration of organic substances expressed as mg. of proteins/ml.

H. Activity of hydrogenase expressed as unit/ml.

T. Activity of thiosulfate reductase expressed as unit$\times 10^{-1}$/ml.

S. Activity of sulfite reductase expressed as unit/ml.

Table I

Distribution of Enzyme Activities in Different Fractions of Extracts Obtained by the Purification Procedure

Fraction	Protein		Sulfite reductase		Thiosulfate reductase		Hydrogenase	
	mg./ml.	total	unit/mg.	total	unit/mg.	total	unit/mg.	total
Precipitate of crude extract	6.6	330	0.34	114	0.83	280	376	125000
Supernatant of crude extract	16.4	1790	0.99	1780	19	33000	140	250000
1st ammonium sulfate fractionation								
Precipitate at 0.33 satn.	27.3	360	0.35	126	19	6700	294	100000
Precipitate between 0.33 and 0.67 satn.	13.0	660	0.71	464	18	12000	48	31500
Dialysed solution of of the latter	9.6	580	0.82	476	16	9000	60	34600
Calcium phosphate gel adsorption								
Eluate with 0.013 *M* phosphate	0.1	1.6	3.8	6	37	58	9	14
Eluate with 0.067 *M* phosphate	0.6	19	4.6	87	41	780	36	690
Eluate with 0.25 *M* phosphate	0.8	17	0.53	9.2	5.4	94	82	140
Supernatant	2.9	290	0.39	113	23	6900	78	23000
2nd ammonium sulfate fractionation								
Precipitate at 0.30 satn.	1.7	2.6	—	—	1.7	4.5	14.1	37
Precipitate between 0.30 and 0.70 satn.	1.6	4.7	7.35	35	82	390	41.3	200

standing. Then 0.51 g. of calcium phosphate gel was added and the gel was collected and eluted successively with 0.013 *M*, 0.067 *M* and 0.25 *M* phosphate buffer, pH 7.0, after washing with water. The eluate with 0.067 *M* buffer which contained a larger part of sulfite reductase was fractionated again by ammonium sulfate precipitation. The activities of sulfite and thiosulfate reductase as well as hydrogenase in each fraction were measured (Table I).

It was indicated that sulfite reductase was purified 7 times compared with that in the supernatant of the crude extract. Although the activity of sulfite reductase did not drop markedly by dialysis or by standing, the yield was not very high in the purification procedure. No increase of the activity was observed when several fractions were brought together.

Table I indicates that sulfite reductase was mainly concentrated in the eluate from the calcium phosphate gel with 0.067 *M* phosphate and thiosulfate reductase remained in the supernatant of the gel adsorption in those conditions. A part of hydrogenase was in the particulate fraction. The different distribution of these three enzymic activities suggests the independent nature of the enzymes.

Similar results were obtained by zone electrophoresis on a starch column (Fig. 2). The results also showed that sulfite reductase, thiosulfate reductase and hydrogenase were different enzymes.

Stoichiometry of Sulfite Reduction—In manometric experiments with the crude preparation, it was shown that sulfite was reduced

Table II

Stoichiometry of Sulfite Reduction

Added sulfite	H_2 uptake	H_2S formed
4.0 μmoles	11.0 μmoles	3.9 μmoles
0	0.1	0.3
Difference	10.9	3.6

1) H_2S absorbed in alkali in the center wells was determined by St. Lorant's method (*23*).

Table III

Effects of Various Substances on Sulfite Reductase and Thiosulfite Reductase

Added Substances	Sulfite reductase		Thiosulfate reductase	
	Concentration	Inhibition	Concentration	Inhibition
α, α'-dipyridyl	$1\times10^{-3}M$	—	$1\times10^{-3}M$	—
KCN	1×10^{-3}	—		
EDTA	1×10^{-3}	—		
	1×10^{-2}	—		
glycine	1×10^{-2}	3%		
oxine	1×10^{-3}	—		
NaF	1×10^{-2}	9	1×10^{-2}	—
NaN_3	1×10^{-3}	2		
	2×10^{-2}	30	1×10^{-2}	50%
thiourea			1×10^{-2}	28
urethan			1×10^{-2}	—
quinacrine	1×10^{-3}	19	1×10^{-3}	80
octyl alcohol			saturated	11
formate	5×10^{-3}	3		
Na_2SeO_4			7×10^{-3}	26
$CuSO_4$	5×10^{-5}	57	5×10^{-5}	100
$NaAsO_2$	1×10^{-3}	67	4×10^{-3}	70
iodoacetate	1×10^{-2}	40		
p-chloromercuribenzoate	4×10^{-4}	70	5×10^{-4}	99
,, + thioglycolate		13		82
Thioglycolate	4×10^{-3}	−35[1]	6×10^{-3}	−6[1]

1) stimulated

to sulfide with an uptake of 3 moles of hydrogen (Table II). The results coincide well with the following equation.

$$Na_2SO_3+3H_2=Na_2S+3H_2O$$

Effects of Various Substances on Sulfite and Thiosulfate Reductase—The arsenite and *p*-chloromercuribenzoate inhibition and the recovery of the activity by thioglycolate shown in Table III lead us to consider that both enzyme systems contain sulfhydryl group (*15*). There is no evidence for the participation of heavy metals in the enzymic activity, because complex forming reagents such as cyanide, α, α'-dipyridyl *etc.* had no or only little effect.

Role of Cytochrome c_3 in Sulfite Reduction—The cell of *Desulfovibrio* contains a large amount of cytochrome, called cytochrome c_3, in spite of its anaerobic nature (*16*, *17*). It was found to play a role of an intermediary electron-carrier in several oxidoreduction. Postgate reported its promoting action on sulfite reduction (*16*). In a previous study (*17*) the authors had failed to observe the necessity of the cytochrome for the reduction of sulfite. However, a positive proof for the necessity of cytochrome c_3 in the sulfite-reducing system was now obtained with the supernatant of ultracentrifugation. The crude extract of the bacteria was centrifuged at $100{,}000\times g$ for 60 minutes and the supernatant was passed through a column of cation-exchange resin, Amberlite IRC 50 Na^+ type. The solution, deprived of the most part of the cytochrome, was employed as the enzyme preparation. It did not absorb hydrogen at all in the presence of sulfite, but by the addition of cytochrome c_3 sulfite

was reduced to sulfide with absorption of 3 moles of hydrogen (Fig. 3). These results indicate probably the role of cytochrome c_3 as an electron carrier between hydrogenase and sulfite reductase system.

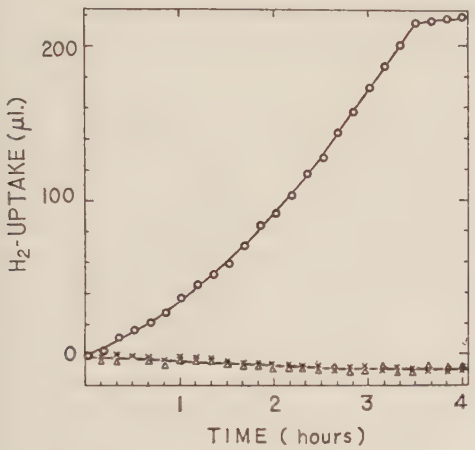

Fig. 3. Effect of cytochrome c_3 on the sulfite reduction.

Complete system contained 12 mg. protein of the extracts deprived of cytochrome c_3, 0.02 μmoles of cytochrome c_3, 3.5 μmoles of Na_2SO_3 and 0.05 *M* phosphate buffer at pH 6.2 to make the final volume of 1.5 ml.

—○— complete system
—△— cytochrome omitted
—×— Na_2SO_3 omitted

Atmosphere H_2, temperature 30°C.

Cytochrome c_3, however, could not replace methylviologen in the system of hydrogenase and the sulfite reductase was partially purified by adsorption with calcium phosphate gel. The crude cell-free extract was passed through a column of Amberlite IRC 50 Na^+ type and treated twice with calcium phosphate gel (75 mg. gel each per 100 mg. protein). The supernatant of the second calcium phosphate gel adsorption and its eluate with 0.25 *M* phosphate buffer (after 48 hours dialysis) were used as enzyme preparations. The supernatant reduced only slowly sulfite with hydrogen in the presence of hydrogenase and the cytochrome in spite of its high activity of sulfite reductase with methylviologen as an electron donor (13 unit /ml.). But the addition of the eluate of the gel which had a low activity (1 unit/ml.) promoted the reduction by several times (Fig. 4). The result indicates the complexity

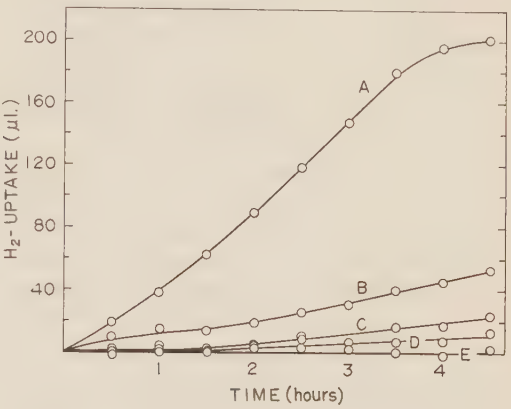

Fig. 4. Reduction of sulfite with hydrogen gas by the reconstructed system.

A, Complete system containing 1 ml. of the supernatant of calcium phosphate-gel adsorption (10.9 mg. protein, 230 unit hydrogenase, 0.003 μmoles cytochrome c_3, 16.1 unit sulfite reductase), 1 ml. of the eluate from calcium phosphate-gel (1.9 mg. protein, 100 unit hydrogenase, no cytochrome, 1.4 unit sulfite reductase), 0.025 μmoles cytochrome c_3, 150 μmoles phosphate buffer, pH 7, 10 μmoles thioglycolate, 0.1 ml. of hydrogenase preparation (2.4 mg. protein, 200 unit hydrogenase) and 4 μmoles sodium sulfite in a total volume of 3.0 ml. Center well contained 0.2 ml. of 2 *N* NaOH. Gas phase was H_2. Temperature: 30°C.
B, The supernatant omitted.
C, The eluate omitted.
D, Cytochrome c_3 omitted.
E, Sodium sulfite omitted.

of the sulfite reducing system in *Desulfovibrio* and the participation of more than two components besides the hydrogenase and the cytochrome in the reaction.

DISCUSSION

The assay methods of sulfite reductase employing methylviologen as electron donor reported above were better than the measurement of evolution of sulfide or decrease of sulfite in the reaction mixture (12). The

devised two methods have their own characteristics. The first one which depends on hydrogen uptake in the presence of hydrogenase and methylviologen is valid in case that the amount of the sulfite reductase is rate-limiting in the reaction. The indicated method was applied under such conditions, although it was inadequate for the study of the effect of pH of the midium and various substances on the reductase.

The second method employing Thunberg tubes to measure the oxidation of reduced methylviologen is not so accurate but simpler. In the case of assay of thiosulfate reductase (*13*), the reduction was carried out at pH 7 and in such conditions the effect of hydrogenase is rather small. The oxidation-reduction potential of methylviologen is lowered when pH is lowered, and at pH 6, where the assay of sulfite reductase is carried out, hydrogen is evolved and the blue color of the reduced form fades completely, if hydrogenase is present. The difficulty was avoided by the provision of carbon monoxide which is a strong inhibitor of hydrogenase (*10*). Carbon monoxide had a marked promoting action on the oxidation of reduced methylviologen by sulfite in the case of sulfite reductase preparation with a little amount of hydrogenase, although the reason was not known.

Lately, carbon monoxide oxidizing enzyme which reduces methylviologen with the oxidation of carbon monoxide to carbon dioxide was found by Yagi in the extract of the bacterium (*18*). In this regard the results obtained for sulfite reductase activity by this method are inaccurate in the crude extracts. As the carbon monoxide oxidinzing enzyme is concentrated in the precipitate at 0.33 saturation of ammonium sulfate in the purification procedure, the assay is reliable in the purified preparation of sulfite reductase.

The reason for the poor yield of the sulfite reductase in the purification may depend partly on the facts that part of the enzyme is in the particulate fraction which dissolves hardly again after precipitation. It is probably true in the first ammonium sulfate precipitation. It is not clear, however, whether the poor recovery in other fractionation procedures was due to the unstability of the enzyme or to partial elimination of some unknown cofactors. In some cases, addition of thioglycolate to the preparation increased the activity but it was not always the case.

The effects of addition of cytochrome c_3 to the cytochrome-free extracts of *Desulfovibrio* to recover the lost reduction activity with hydrogen had been observed in the case of thiosulfate (*19*), colloidal sulfur (20), hydroxylamine (*20*, *21*), and nitro-compounds* as well as of adenosine-5′-phosphosulfate (*22*) or sulfate in the presence of of ATP (*5*, *21*). In the case of sulfite, the authors had failed to find positive effect using the crude extract containing small particles. As reported above, the effect of the cytochrome was observed with the supernatant of ultracentrifugation. The sedimented particles contained a large amount of cytochrome and sulfite reductase. They may be situated closely on the particles to work directly and cover the effect of addition of external cytochrome to the soluble system.

The role of cytochrome in sulfite reduction is not clear. The reduced cytochrome was oxidized spontaneously by colloidal sulfur, hydroxylamine and nitro-compounds (*10*) and enzymically by adenosine-5′-phosphosulfate as well as by sulfate in the presence of ATP (*22*). It is clear that the cytochrome is the intermediary electron carrier between hydrogenase and the acceptors directly or through adenosine-5′-phosphosulfate reductase. In the case of thiosulfate reduction, as the oxidation-reduction potential of the system S_2O_3-SO_3+H_2S (−0.423 volt) is much lower than that of cytochrome c_3 (−0.205 volt), a possibility remains that the oxidation of the cytochrome by thiosulfate was not observed in the extract (*17*). In the case of sulfite, at first sight, the cytochrome may be regarded

* Ishimoto, M., and Shiraki, M., unpublished data.

similarly as an intermediary electron carrier. Sulfite reductase may accept electrons from the cytochrome not directly but through other proteins. From this point of view, however, it is difficult to explain the reason why the reduced cytochrome in the crude extract as well as in the supernatant of ultracentrifugation was oxidized by the addition of sulfite only very slowly (*22*). There is another possibility that several reductases are necessary in the reduction of sulfite, since the reduction of sulfite to sulfide is accompanied by an elimination of three atoms of oxygen, each of which is reduced by two electrons. Therefore, if the two-electron reduction is presumed to take place, there must occur three steps of reduction, the nature of which is a matter of further investigation. At any rate, it is sure that the enzyme system of sulfite reduction is of a complex nature and further study is necessary to elucidate the mechanism.

SUMMARY

Assay methods for the estimation of sulfite reductase were devised with methylviologen as an electron donor. Sulfite reductase in the extracts of *Desulfovibrio* was purified partially and its properties were investigated. Cytochrome c_3 was necessary in the reduction of sulfite with hydrogen, while other components besides the sulfite reductase was needed for the sulfite reduction when the cytochrome was employed as an intermediary electron carrier instead of methylviologen.

The authors thank Prof. S. Akabori and Prof. F. Egami for their valuable advices.

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Action of Trypsin upon Glutamic Acid-γ-hydrazide Derivative*

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It has been repeatedly demonstrated that trypsin hydrolyses peptide, amide or ester linkages of lysyl and arginyl compounds (*1*, *2*). Recently it has been shown that benzoyl-L-histidine ester (*3*), *n*-fatty acid esters (*4*), ε-aminocaproyl compounds (*5–7*) and esters of benzoylhomoarginine and benzoyldiaminoisobutyric acid (*8*, *9*) are also susceptible to the action of the enzyme. The author has found in a course of the study on tryptic hydrolysis of lysine analogus compound that glutamic acid-γ-hydrazide derivative is hydrolysed slowly by the action of the enzyme.

$NH_2CH_2CH_2CH_2CH_2CHCOOH$
NH_2

lysine

$NH_2NHOCCH_2CH_2CHCOOH$
NH_2

Glutamic acid-γ-hydrazide

The present communication deals with detailed studies concerning the hydrolysis of the glutamyl-γ-hydrazide compounds by the action of trypsin.

EXPERIMENTAL

The rate of disappearance of the acid ester was measured by the hydroxamic acid method of Hestrin (*10*). The rate of cleavage of the acid amide and peptide was followed by measurement of the formation of ammonia and free amino group by the ninhydrin method of Moore and Stein (*11*). Except where otherwise noted, hydrolysis rate was assayed as follows: To 2ml. of 0.01–0.1 *M* substrate solution 2 ml. of 200–400 μg./ml. trypsin solution was added. Then the mixture was incubated at 30°C. In all cases substrate and trypsin were dissolved in 0.1 *M* phosphate buffer except for the experiments on the effect of pH. At intervals 1 ml. of the mixture was taken and the hydrolysis rate of the substrate was analyzed by the method as described above. Further, in all cases, control experiments were made to determine the lability of the substrate, in the absence of the enzyme to be added, at the same pH and temperature. Where no experimental data are given for such control experiments, the substrates were found to be stable in the course of the experiments.

Tryptic cleavage product of carbobenzoxy-L,γ-hydrazinoglutamyl-L,γ-hydrazinoglutamyl-L-alanine was isolated as follows: To 5ml. of 0.5% solution of the compound (pH 7.4) 0.5mg. of trypsin was added, and the mixture was incubated at 30°C for 24 hours. After incubation the mixture was dried *in vacuo*, and the cleavage product was identified by paper chromatographic analysis using a solvent mixture, phenol—water (7 : 3).

Twice recrystallized trypsin purchased from the Pentex Inc., Illinois, was used as the enzyme.

Carbobenzoxy-L-glutamic Acid-α-ethyl Ester-γ-hydrazide Hydrochloride—Carbobenzoxy-L-glutamic acid-γ-hydrazide was esterified twice in ethanol saturated with HCl at 5°C, yield, 72 per cent; m.p., 192°C; $[\alpha]_D^{20}$ —16.5°C (2 per cent in water). $C_{15}H_{22}O_5N_3Cl$ (259.79). Calculated, N 11.68; found, N 11.46.

Carbobenzoxy--L-isoglutamine-γ-hydrazide—Carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide hydrochloride was converted to the amide. Yield, 56 per cent; m.p., 136°C; $[\alpha]_D^{20}$ —4.5°C (2 per cent in water). $C_{13}H_{18}O_4N_4$ (293.23). Calculated, N 19.04; found, N 18.92.

Carbobenzoxy-L, γ-hydrazinoglutamyl-L, γ-hydrazinoglutamyl-L-alanine—This compound was prepared by the treatment of carbobenzoxy-L,γ-methylglutamyl-L-glutaminyl-L-alanine with excess amount of hydrazine hydrate. The starting tripeptide was kindly supplied from Dr. T. Shiba of the Laboratory of Organic Chemistry of our University.

Polyglutamic Acid-γ-hydrazide—These compounds were prepared by the treatment of polyglutamic acid-γ-methyl ester with excess amount of anhydrous hydrazine at room temperature. The L- and D-polymer of glutamic acid-γ-methyl ester were kindly supplied

* This work was presented at the 32th Annual Meeting of the Japanese Biochemical Society at Osaka, November, 1959.

Prof. J. Noguchi, Kanazawa University.

	N-Analysis	$[\alpha]_D^{20}$
L-Polymer (γ-hydrazide)	21.0 per cent	−89.8
D-Polymer (γ-hydrazide)	17.6	+54.5

RESULTS

Behavior of Trypsin upon Glutamic Acid-γ-hydrazide Derivative—In Table I are presented the data indicating that glutamic acid-γ-hydrazide derivatives can be hydrolysed to some extent by the action of trypsin. Much more extensive hydrolysis was observed with both compounds listed in the table if a longer time was employed under the same conditions. These compounds seem to suffer hydrolysis at one position of ester or amide linkage as evidenced by paper chromatographic detection of carbobenzoxyglutamic acid-γ-hydrazide in the product.

Effect of pH on the hydrolysis of glutamic acid-γ-hydrazide derivatives by the enzyme action was investigated as shown in Fig. 1. The optimnm pH was found to be 7.3–7.5 and 7.5, respectively, for carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide and carbobenzoxy-L-isoglutamine-γ-hydrazide. The affinity of the enzyme to these compounds was determined at optimum pH by the Lineweaver-Burk graphical treatment (Fig. 2). The Michaelis constant Km was $71\times10^{-3}M$ for carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide, and $24\times10^{-3}M$ for carbobenzoxy-L-isoglutamine-γ-hydrazide.

As can be seen from Table II it was found that the hydrolysis of glutamic acid-γ-hydrazide derivatives by trypsin follows the kinetics of the first order reaction and that the velocity constant is proportional to the enzyme concentration. The proteolytic coefficient k_3 was estimated to be 49×10^{-3} with carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide, and 21×10^{-3} with carbobenzoxy-L-isoglutamine-γ-hydrazide, as shown in Table II.

Tryptic Hydrolysis of Peptides Containing Glutamic Acid-γ-hydrazide—A search for the cleavage products of carbobenzoxy-L,γ-hydrazinoglutamyl-L-alanine by paper partition chromatography revealed that a small amount of glutamic acid-γ-hydrazide and carbobenzoxyglutamic acid-γ-hydrazide, together with relatively large amount of free alanine and carbobenzoxyglutamylglutamic acid-γ,γ′-dihydrazide were formed from the substrate (Fig. 3). A trace of an unknown compound was found as a product as shown in Fig. 3.

TABLE I

Action of Trypsin on Glutamic Acid-γ-hydrazide Derivatives

Substrate	Concentration		pH	Hydrolysis (%) at 30°C					
	Trypsin	Substrate		30	60	90	120	180	240 (min.)
	μg./ml.	M							
Carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide hydrochloride	200	0.005	7.8	13	32	32	36	47	51
	100	0.01	7.6		10				
	100	0.005	5.6	0	0.2	1	3	4	8
	115	0.05	7.3		12		20		
	200	0.0045	7.3	7	10		11		12
Carbobenzoxy-L-isoglutamic-γ-hydrazide	100	0.005	7.6		5.5				
	100	0.015	7.3		6.9				

In all cases 0.1 M phosphate buffer was used as a medium.

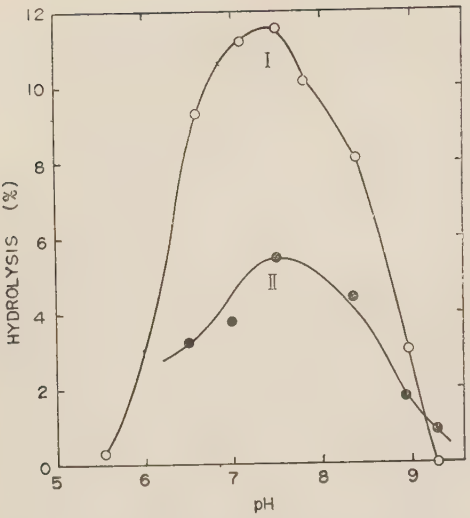

FIG. 1. Effect of pH on the hydrolysis of glutamic acid-γ-hydrazide derivatives.

I: Carbobenzoxy-L-glutamic acid-α-hydrazide hydrochloride, at 37°C.

II: Carbobenzoxy-L-isoglutamine-γ-hydrazide, at 30°C.

Buffers: pH 5–8, phosphate buffer; pH 8–10, borax buffer (0.05 M). Substrate concentration was 0.01 M in buffer solution, and in each reaction was involved 100 μg./ml. of trypsin. Incubation was made for 60 minutes.

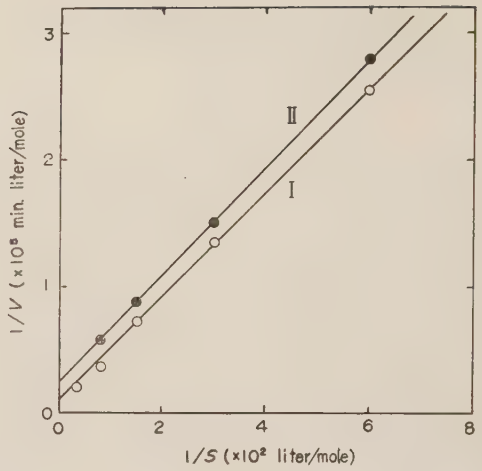

FIG. 2. Influence of concentration of glutamic acid-γ-hydrazide derivatives on the rate of reaction.

I: Carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide hydrochloride.

II: Carbobenzoxy-L-isoglutamine-γ-hydrazide.

Each reaction mixture contained 100 μg./ml. of trypsin in 0.1 M phosphate buffer (pH 7.3). Incubation, at 30°C for 60 minutes.

TABLE II

Proteolytic Coefficient of Glutamic Acid-γ-hydrazide Derivatives

Substrate	Enzyme concentration (protein N μg./ml.)	Reaction velocity constant K (10^4)	Proteolylic coefficient k_3 (10^3)	
Carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide hydrochloride	17.9	8.08	46.9	48.6
	14.3	7.08	49.5	
	11.4	5.46	47.9	
	8.6	4.46	51.8	
Carbobenzoxy-L-isoglutamine-γ-hydrazide	15.0	3.10	20.7	

Substrate concentration was 0.05 M in 0.1 M phosphate buffer (pH 7.3), and contained a definite amount of trypsin as described in the table. Incubation was made for 10, 20, 30, 40, 60 and 120 minutes at 30°C. The reaction velocity constant K was calulated as a first order reaction. The k_3 is expressed in moles/liter/min./mg. enzyme-N/ml.

This was not yet identified clearly although it gave only free glutamic acid by hydrolysis with 6 N hydrochloric acid. Qualitatively identical results were obtained from the other chromatograms using a different solvent mixture, n-butanol, acetic acid and water. No spot was observed when control samples without either substrate or enzyme were analysed.

Further the tryptic cleavage of the syn-

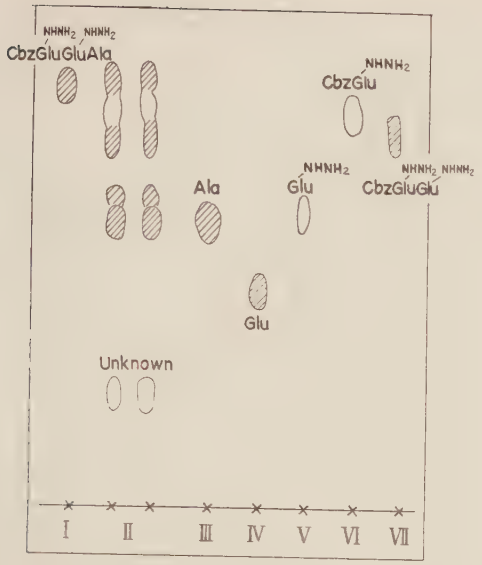

Fig. 3. A paper chromatogram of the hydrolytic product of carbobenzoxy-L,γ-hydrazinoglutamyl-L,γ-hydrazinoglutamyl-L,-alanine.

I: Carbobenzoxy-L,γ-hydrazinolutamyl-L,γ-hydrazinoglutamyl-L-alanine (substrate).

II: Tryptic cleavage product of I (left, 18 hours incubation, right, 24 hours incubation.

III: L-Alanine.

IV: L-Glutamic acid.

V: L-Glutamic acid-γ-hydrazide.

VI: Carbobenzoxy-L-glutamic acid-γ-hydrazide.

VII: Carbobenzoxy-L-glutamyl-L-glutamic acid-γ,γ'-di-hydrazide.

Solvent system: phenol-water (7:3).

The detection was performed by spraying ninhydrin and ammonia-silver nitrate reagents.

thetic hydrazine-treated polyglutamic acid-γ-methyl esters was examined as presented in Table III. It is evident that the polymer or L-glutamic acid-γ-hydrazide was partly hydrolysed at relatively high concentrations of the enzyme, althongh the hydrolysis was not so advanced at 22 hours. Qualitative paper chromatographic and electrophoretic examination of the cleavage products released by the trypsin showed that four small but definite spots reacted with the ninhydrin and the ammonia-silver nitrate reagents, which were not yet identified. The D-polymer was completely resistant to the action of the enzyme.

Table III

Action of Trypsin on Polyglutamic Acid-γ-hydrazides

Incubation time	Trypsin		Hydrolysis (%)	
	Concentration	Ratio to substrafe	L-Polymer	D-Polymer
hrs.	μg./ml.			
6	50	1:100	0.3	0
22	100	1: 50	2.3	0
51	150	1: 30	2.6	0

Polymer, 0.5% solution in 0.1 *M* phosphate buffer, pH, 7.4; Temperature, 30°C.

Inhibition of Trypsin by Glutamic Acid-γ-hydrazide Derivative—Influence of glutamic acid-γ-hydrazide derivative upon the tryptic hydrolysis of esters and amides of α-benzoyl-L-lysine or -L-arginine was investigated as shown in Table IV. It is obvious that the tryptic attack was not inhibited even at re-

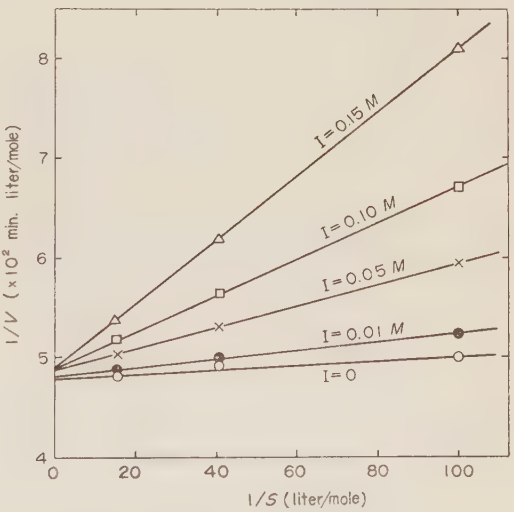

Fig. 4. Competitive inhibition of trypsin by carbobenzoxy-L-glutamic acid-γ-hydrazide.

Each reaction mixture contained α-benzoyl-L-lysine ethyl ester (substrate, concentration 0.05 *M*), carbobenzoxy-L-glutamic acid-γ-hydrazide (inhibitor, concentration, I, as described in the figure), and contained 50 μg./ml. of trypsin in 0.1 *M* phosphate buffer (pH 7.6). Incubation was carried out at 30°C for 5–15 minutes.

latively high concentrations of the glutamic

TABLE IV

Influence of Glutamic Acid-γ-hydrazide Derivatives upon the Tryptic Hydrolysis of Benzoyllysine and Benzoylarginine Derivatives

Inhibitor	Concentration *M*	Inhibition (%) Substrate: α-Benzoyl-L-lysine ethyl ester 0.05 *M*	Substrate: α-Benzoyl-L-arginine ethyl ester 0.05 *M*	Substrate: α-Benzoyl-L-argininamide 0.05 *M*
α-Benzoyl-L-arginine	0.05	3–4	0–3	42–53
α-Benzoyl-L-arginine ethyl ester (hydrochloride)	0.05	—	—	85–99
α-Benzoyl-L-argininamide hydrochloride (monohydrate)	0.05	3–5	2–3	—
Carbobenzoxy-L-glutamic acid-γ-hydrazide	0.05	14	—	—
	0.01	5	—	6
Carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide (hydrochloride)	0.1— 0.01	—	—	0
Carbobenzoxy-L-isoglutamine-γ-hydrazide	0.01	0	—	0

Each reaction mixture contained 100 μg./ml. of trypsin and definite amounts of the substrate and the inhibitor (concentrations, as described in the table), in a final volume of 1.5 ml. of 0.1 *M* phosphate buffer, pH 7.6. Incubation was carried out for 60 minutes at 30°C.

acid-γ-hydrazide derivative except in the case of carbobenzoxyglutamic acid-γ-hydrazide. Addition of this acid induced some inhibition on the rate of the tryptic hydrolysis of benzoyl-L-lysine ethyl ester and benzoyl-L-argininamide.

The kinetics of the inhibition of tryptic activity by carbobenzoxy-L-glutamic acid-γ-hydrazide were studied, with α-benzoyl-L-lysine ester as a substrate. Fig. 4 shows the inhibition of the tryptic activity under several concentrations of the inhibitor. The data are plotted with the reciprocal of the reaction velocity as the usual treatment. The results revealed that this compound practically inhibited the activity of trypsin in a competitive fashion. The inhibition constant *Ki* was estimated to be 12×10^{-3} *M*.

DISCUSSION

The present study indicates that both the ester and amide of L-glutamyl-γ-hydrazide compound are hydrolysed in part by the action of trypsin. It is known that optimum pH of tryptic hydrolysis of usual synthetic substrates of the enzyme, α-benzoyl-L-argininamide and α-benzoyl-L-lysinamide, is at 7.8 (*12*). The optimum pH of the hydrolysis of glutamic acid-γ-hydrazide derivatives was observed to be at pH 7.3–7.5 (Fig. 1). This very minor deviation in optimum pH might be due to differences among the ω-amino group and γ-hydrazino group.

Michaelis constant (*Km*) for the hydrolysis of the glutamic acid-γ-hydrazide compounds was found to be $24–71\times10^{-3}$ *M*, a value remarkably higher than the *Km* for the arginyl, lysyl and ε-aminocaproyl compounds (*7*). This indicates that the affinity of trypsin to the glutamic acid-γ-hydrazide derivative is smaller than that to the arginyl, lysyl and ε-aminocaproyl compounds.

The proteolytic coefficient k_3 for tryptic hydrolysis of carbobenzoxy-L-isoglutamine-γ-hydrazide was found to be 21×10^{-3} and this value is about half of that for α-benzoyl-L-argininamide (38×10^{-3}) (*13*). The k_3 for the hydrolysis of carbobenzoxy-L-glutamic acid-α-ethyl ester-γ-hydrazide is 49×10^{-3} and this value is about 1/6 of that for α-benzoyl-L-arginine ethyl ester (280×10^{-3}) (*14*, *15*).

It was also found that the peptide and polymer containing glutamic acid-γ-hydrazide were hydrolysed very slowly by trypsin as compared with protein and poly-L-lysine (*16*), as shown in Fig. 3 and Table III. This phenomenon also indicates that the affinity of the enzyme to the synthetic peptide and polymer is smaller than that to the natural substrate such as protein and polylysine, although glutamic acid-γ-hydrazide has a molecular structure similar to that of lysine. Transpeptidation of γ-hydrazinoglutamyl residue by trypsin could not be observed in the hydrolysis of the polymer of L-glutamic acid-γ-hydrazide.

The inhibition of the rate of the tryptic hydrolysis of usual synthetic substrate by glutamic acid-γ-hydrazide and its derivative is smaller than that by the arginyl compound (Table IV). However, since ornithyl and δ-aminovaleryl compounds did not inhibit competitively the tryptic hydrolysis (*7*), it is concluded that glutamic acid-γ-hydrazide and its derivative have a little affinity to the enzyme.

SUMMARY

Ester and amide of carbobenzoxy-L-glutamic acid-γ-hydrazide, a lysine analogous compounds, have been synthesized and tested their nature as substrate for trypsin. These compounds can be partly hydrolysed by the enzyme action. Optimum pH, *Km* and proteolytic coefficient k_3 were also determined for the hydrolysis. Peptide and polymer containing L-glutamic acid-γ-hydrazide residues were also slowly hydrolysed by the action of the enzyme, but those containing D-isomer were not. The inhibition of the tryptic activity npon usual substrate by glutamic acid-γ-hydrazide compounds were studied.

The author wishes to express his hearty thanks to Prof. S. Akabori and Dr. A. Tsugita of our University, for their interest and encouragement throughout the course of this work.

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Studies on the Distribution and Phosphate-Turnover of the Acid-Soluble Phosphorus Compounds in Various Normal and Neoplastic Tissues of Rats

III. Chromatographic Isolation and Identification of Nitrogen-Containing Phosphate Esters

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In the previous papers (*1*, *2*), the authors described the Dowex 1 formate unadsorbable radioactivity in the perchloric acid extracts from tissues of rats injected with P^{32}-inorganic phosphate. This radioactivity was high in liver, spleen, thymus, tumor *etc.*, but low in muscle and heart, and could be adsorbed on Dowex 50 column, eluted with diluted HCl and resolved into two peaks.

In this study, this radioactivity was further analysed and identified as *o*-phosphoryl ethanolamine and phosphoryl choline. CDP-ethanolamine* and CDP-choline showing practically no radioactivity were also isolated from the Dowex 1 formate unadsorbable fraction. On the other hand, synthetic *o*-phosphoryl serine was found to be adsorbed on Dowex 1 formate and eluted in almost the same position as the radioactive peak B in liver, which appeared closely next to AMP (*1*). But appreciable amounts of *o*-phosphoryl serine could not be detected from the liver fraction. The high radioactive peak B in muscle on the Dowex 1 formate chromatogram (*2*), which also appeared next to AMP, was isolated and identified as phosphocreatine.

Thus the differences in the phosphate incorporation into the nitrogen-containing phosphoric esters in various rat tissues was studied. At the same time, a chromatographic method for isolation of nitrogen-containing phosphoric esters was presented.

The details of the experiments will be described in this paper.

* abbreviations;
AMP: adenosine 5′-phosphate
CMP: cytidine 5′-phosphate
CDP-ethanolamine: cytidine diphosphate-ethanolamine
CDP-choline: cytidine diphosphate-choline

EXPERIMENTALS

Preparation of Perchloric Acid Extracts—For analytical purposes, 1 to 3 g. of the tissue was frozen quickly in acetone-dry ice, crushed in an ice cold porcelain mortar with a small amount of dry ice. The crushed pieces were immediately homogenized with two volumes of ice cold 0.6 *N* perchloric acid in a glass homogenizer. The homogenate was centrifuged and the supernatant was removed. The precipitate was washed once with two volumes of 0.2 *N* perchloric acid by centrifuging. The supernatant and the washing were combined, added with a few drops of 0.2% phenol red solution, cooled in ice water, and then cautiously neutralized first with 30% KOH and then with 1 *N* KOH. The resulting precipitate was centrifuged, and the supernatant was stored in a refrigerator for two hours. The small amount of precipitate formed after 2 hours was separated by centrifuging or decantation, and the final supernatant was immediately applied to the analysis.

For preparative purposes, 120 g. of rat liver was immediately frozen in acetone-dry ice. To each 30 g. of the frozen tissue, 60 ml. of 0.6 *N* perchloric acid was added, and homogenized in an ice cold Waring blendor. The combined homogenate was centrifuged and the supernatant was removed. The precipitate was washed once with 60 ml. of 0.2 *N* perchloric acid by centrifuging. The supernatant and the washing were combined and neutralized as described above.

Preparation of Ion Exchange Columns—Dowex 1 chloride resin (X10, 200–400 mesh) was converted into formate form. The formate columns were throughly washed before use as described previously (*1*). For analytical purposes 0.6×40 cm. columns, and for preparative purposes 2.2×50 cm. columns were used.

Dowex 50 resin (X4, 200–400 mesh) was repeatedly treated with 6 *N* HCl, and then washed with large volumes of water until the washings showed no acidic reaction. For analytical purposes 0.6×45 cm. columns, and for preparative purposes either 4×25 cm. or 1.2×120 cm. columns were used.

Analysis of the Samples Collected—The U.V. absorption was measured with a Hitachi Spectrophotometer (type EPU-2A). Radioactivity of P^{32} was measured in an usual way using counting plates with aliquots of the fractions. *o*-Phosphoryl ethanolamine and *o*-phosphoryl serine were determined with ninhydrin reaction according to the method of Yemm and Cocking (*3*). For the determination of phosphoryl choline, the samples were hydrolysed with 6 *N* HCl (100°C, 24 hours), dried in a vacuum desiccator over NaOH. The resulting free choline was dissolved in 2 ml. of of water, and precipitated with the addition of 0.2 ml. of the Roman reagent (*4*). The choline periodide precipitate formed was centrifuged down, dissolved in 3 ml. of ethylene dichloride, and the absorption at 365 mμ was measured. This photometric method for the determination of free choline was essentially based on the method of Levy *et al.* (*5*).

Paper Chromatography—The Toyo Filter Paper No. 53 and, in some cases, the Whatman No. 1 were used. Most of the experiments were carried out in ascending technique. The solvent mixtures chiefly employed were as follows:

a) Methanol-Formic Acid-Water (80 : 15 : 5) (*6*)
80 volumes of methanol, 15 volumes of concentrated (85 per cent) formic acid, and 5 volumes of water.

b) Methanol-Ammonia-Water (60 : 10 : 30) (*6*)
60 volumes of methanol, 10 volumes of concentrated (28 per cent) ammonia, and 30 volumes of water.

c) *tert*-Butanol-Picric Acid-Water (80 : 2 : 20) (*7*)
80 ml. of *tert*-butanol, 2 g. of picric acid, and 20 ml. of water.

d) *n*-Butyric Acid-NaOH-Water (69 : 0.85 : 31) (*8*)
0.85 g. of NaOH was dissolved in 100 ml. of 69% (v/v) *n*-butyric acid.

e) Phenol-Water (*72* : 28) (*9*)

f) Phenol saturated with Water (in acetic acid atmosphere)

g) *n*-Butanol-Acetic Acid-Water (4 : 1 : 1)
4 volumes of *n*-butanol, one volume of glacial acetic acid, and one volume of water.

h) Ethanol-Ammonia (95 : 5) (*10*)
95 volumes of 95% ethanol and 5 volumes of 28% ammonia.

i) *n*-Butanol-Acetic Acid-Water (100 : 30 : 85) (*10*)

j) *n*-Propanol-Pyridine-Water (4 : 1 : 1)

k) Acetone-Trichloracetic Acid (25%) (75 : 25) (*11*)

l) Methylethyl Ketone-Formic Acid-Water (7:1:2)

m) *n*-Butanol-Formic Acid-Water (4 : 1 : 1)

n) *n*-Propanol-Acetic Acid-Water (4 : 1 : 1)

For the detection of phosphorus compounds on the paper, Hanes and Isherwood's molybdate reagent (*7*) was employed. After spraying this reagent, the paper was dried by air stream, and the resulting phosphomolybdate was reduced by illuminating with low pressure mercury arc. For the detection of U.V. absorbing materials on the paper, a commercial mercury arc with a glass U.V. filter was employed. The U.V. absorbing spots were easily detected with the aid of a fluorescent plate. Amino compounds were detected with 0.1% ninhydrin in *n*-butanol. For the detection of free choline, the Levine-Chargaff's reagent was used (*12*). Creatine was detected, after converting into creatinine by heating at 110°C for 3 hours, with spraying 2 *N* NaOH followed by saturated picric acid.

Authentic Samples—*o*-Phosphoryl ethanolamine and *o*-phosphoryl serine were obtained from the Nutritional Biochemicals Corporation (Cleveland, Ohio). Phosphoryl choline, cytidine diphosphate choline, and cytidine diphosphate were obtained from the Sigma Chemical Company (St. Louis, Missouri). Cytidine, cytidine-5′-phosphate were supplied from the Takeda Pharmaceutical Industries, Ltd. (Osaka).

RESULTS

Separation and Isolation of the Dowex 1 Formate Unadsorbable Phosphorus Compounds—Fig. 1 illustrates a Dowex 1 formate chromatogram of the acid soluble fraction from rat liver obtained one hour after the intraperitoneal injection of P^{32} inorganic phosphate (*1*). Peak A in this figure is the Dowex 1 formate unadsorbable radioactivity. In most of the tissues, the peak A showed 2 to 5 per cent of the total acid soluble radioactivity, but in muscle and heart the peak A was very small and showed less than 1 per cent of the total (Table I). The peak A was found to be adsorbed on Dowex 50 column almost quantitatively.

Fig. 2 shows the result of the gradient

elution of the adsorbed radioactivity from the Dowex 50 column. Two radioactive components and three U.V. absoabing components are observed in this figure. In this experimental case, A_1 was larger than A_2 but this was not a regular result but in many other cases A_2 was larger than A_1. Physiological or nutritional conditions might affect the amounts of these radioactivities but any precise study was not carried out.

Thymus, spleen, and hepatoma were also tested for A_1 and A_2, and found that all of them contained these two peaks, which were proved to be identical with those in liver by tests with paper chromatography. The U.V. absorbing peaks (k_1 and k_2) in liver were seemed to show more constant relative amounts. And also these relative amounts varied from tissue to tissue.

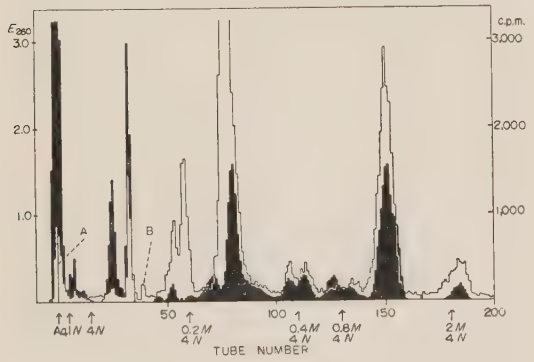

FIG. 1. Chromatography of the acid-soluble phosphorus compounds in rat liver. (Wet weight: 2.7 g.)

The animal was sacrificed one hour after the intraperitoneal injection of P^{32} inorganic phosphate (60 μc).
column: Dowex 1 formate, 0.6×40 cm.
mixer volume: 150 ml.
Fractions were collected for each 4 ml.
Arrows indicate the positions where the solution in the reservoir was changed. For details, see previous paper (*1*).
black peaks: U.V. absorption
colorless peaks: radioactivity

These five components (A_1, A_2, k_1, k_2, and k_3) were separated from the liver extract and purified by repeated column chromatography. Purified A_1 and A_2 had no U.V. absorption

TABLE I

Radioactivity of Fractionation A

One hour after the intraperitoneal injection of 60μc of P^{32} inorganic phosphate

tissue	total acid-soluble phosphates c.p.m./g. wet tissue	fraction A c.p.m./g. wet tissue	%
striated muscle	26,500	121	0.46
heart	34,550	320	0.93
liver	35,750	1,168	3.3
thymus	37,380	1,535	4.1
spleen	23,150	1,573	6.8
lung	15,890	899	5.7
fetal liver	3,465	150	4.4
ascites hepatoma (solid type, AH 7974)	47,700	1,675	3.5
ascites hepatoma (,, ,, local irradiation of X-ray, 1000 r)	8,100	230	2.7
ascites hepatoma (solid type, AH 130)	24,950	597	2.4
ascites hepatoma (,, ,, local irradiation of X-ray, 1000 r)	10,990	463	4.2

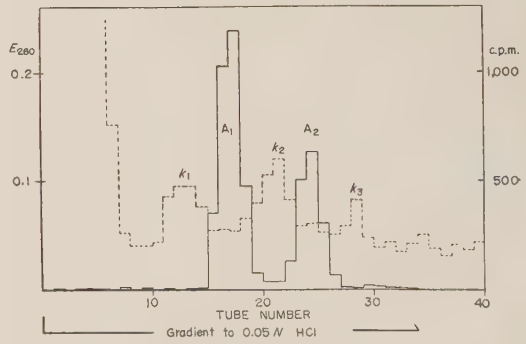

FIG. 2. Chromatography of the Dowex 1 formate unadsorbable fraction.

Wistar-King A rat, female, body weight 82 g. (110 days of age). One hour after the intraperitoneal injection of 40μc of P^{32} inorganic phosphate. liver tissue analysed: 2.2 g. (total liver weight 3.5 g.)
column: Dowex 50 (X4, 200–400 mesh, -H form) 0.6×40 cm.
mixer volume for gradient elution: 150 ml.
velocity of elution: 8 ml./hour
volume of fractions collected: each 4 ml.
0.5 ml. from each fraction was used for the radioactivity measurement.
solid line: radioactivity, dotted line: U.V. absorption.

and showed a tendency to crystallize from the highly concentrated solution in a vacuum desiccator. Purified k_1, k_2, and k_3 had no measurable radioactivity. Scheme I illustrates the procedure employed for the isolation of A_1 and A_2. Fig. 3 shows the results of the 1st preparative chromatography with Dowex 50 in Scheme I. Later, Yemm and Cocking's ninhydrin method was used for the isolation of A_1.

k_1, k_2, and k_3 were prepared in almost the same way as Scheme I. The amounts of these three were much less than A_1 and A_2.

Identification of Phosphoryl Ethanolamine and Phosphoryl Choline—Characteristics of purified A_1 and A_2 on paper chromatography are shown in Table II. A_1 and A_2 showed similar R_f values with many solvents but with the phenol solvent A_2 showed much larger R_f value than A_1. A_1 was positive for ninhydrin spot test but A_2 was negative.

On hydrolysis with 6 *N* HCl at 100°C for 48 hours, A_1 liberated inorganic phosphate and at least three ninhydrin positive spots.

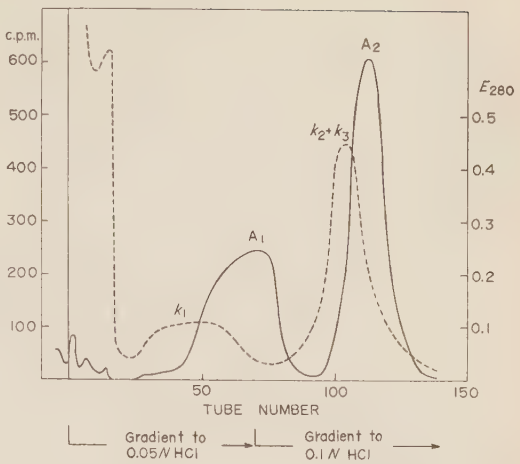

Fig. 3. Preparative chromatography of the Dowex 1 formate unadsorbable phoshporus compounds in liver.

200 g. of liver tissue was obtained from 25 rats. 5 of them were injected with 100 μc of P^{32} inorganic phosphate intraperitoneally, sacrificed one hour after the injection. The PCA extract (600 ml.) was passed through Dowex 1 formate column (2.2×50 cm.), adsorbed on Dowex 50 (X4, -H form, 4×25 cm.) column.
elution: gradient to 0.05 *N* and then 0.1 *N* HCl (mixer volume 250 ml. 25 ml./hour)
fractions collected: each 8 ml.
sold line: radioactivity; dotted line: U.V. absorption.

Scheme I

Purification of A_1 and A_2

25 Rats, Liver 200 g. (5 rats were injected with 100μc of P^{32} inorganic phosphate intraperitoneally, sacrificed 1 hour after the injection)

↓

Freezing in Acetone-Dry Ice

↓

Homogenization in PCA

↓

PCA Extract 600 ml.

↓

Passed through Dowex 1 Formate (X10, 2.2×50 cm.)

↓

1st Adsorption on Dowex 50 (X4, -H Form, 4×25 cm.)

↓

Gradient Elution with dil. HCl

Crude Fr. A_1 (96,240 c.p.m.)	Crude Fr. A_2 (131,470 c.p.m.)
↓ Lyophilization	↓ ,,
2nd Adsorption on Dowex 50 (0.6×40 cm.)	,,
↓ Gradient Elution and Lyophilization	,,
Fr. A_1 (79,500 c.p.m.)	Fr. A_2 (88,800 c.p.m.)
↓ 3rd Adsorption on Dowex 50 (0.6×40 cm.)	,,
↓ Gradient Elution and Lyophilization	,,
↓ *Purified Fr. A_1* (75,500 c.p.m.)	↓ *Purified Fr. A_2* (77,300 c.p.m.)

So, in the previovs paper (*1*), it was persumed that A_1 might be a phosphopeptide. However, on further investigation, A_1 showed the same R_f value as *o*-phosphoryl ethanolamine with all kinds of solvent tested. The solvent tested were as follows (see also description under Methods):

n-Butyric Acid-NaOH-Water; Phenol saturated with Water (acetic acid atmosphere); *n*-Propanol-Pyridine-Water; Methanol-Formic Acid-Water; Methylethyl Ketone-Formic Acid-Water; *n*-Butanol-Formic Acid-Water; Methanol-Ammonia-Water; *n*-Propanol-Acetic Acid-Water; Phenol-Water (72 : 28); *n*-Butanol-Acetic Acid-Water; Acetone-Trichloracetic Acid; *tert*-Butanol-Picric Acid-Water

Furthermore, A_1 and authentic phosphoryl ethanolamine was hydrolysed under the same condition and it was found that they produced the same ninhydrin positive spots. This was checked with three solvent systems as follows:

n-Butanol-Acetic Acid-Water; *n*-Propanol-Acetic Acid-Water; Phenol saturated with Water (acetic acid atmosphere)

Later, it was found that, when treated with 6 *N* HCl at 100°C for 24 hours, ethanolamine itself produced three or four ninhydrin positive spots on the paper chromatography with *n*-Butanol-Acetic Acid-Water (4 : 1 : 1).

Thus A_1 was identified as *o*-phosphoryl ethanolamine.

A_2 liberated inorganic phosphate and a positive spot for Levine-Chargaff's phosphomolybdate reagent when hydrolysed with 6 *N* HCl at 100°C for 60 hours. This spot showed the same R_f values as choline. The solvent tested were as follows.

n-Butanol-Acetic Acid-Water (4 : 1 : 1); *n*-Butanol-Acetic Acid-Water (100 : 30 : 85); Ethanol-Ammonia; Methanol-Ammonia-Water; Phenol-Water (72 : 28)

Then authentic phosphoryl choline and A_2 were compared and found they had the same R_f values with variety of solvent systems.

Table II

Paper Chromatography of Purified A_1 and A_2

	Solvent	R_f	Spot Test for Phosphates (Hanes-Isherwood)	Radio-activity	Ninhydrin Spot Test	Number of Spot	R_f of the Authentic Compound
							(Phosphoryl Ethanolamine)
A_1	Methanol-conc. NH_3-Water (60 : 10 : 30)	0.59	(+)	(+)	(+)	1	0.59
A_1	*tert*-Butanol-Picric Acid-Water (80 : 2 : 20)	0.29	(+)	(+)	(/)	1	0.29
A_1	*n*-Butanol-Acetic Acid-Water (4 : 1 : 1)	0.12	(+)	(+)	(+)	1	0.12
A_1	*tert*-Amyl Alcohol-Acetic Acid-Water (5 : 2 : 3)	0.35	(+)	(+)	(+)	1	/
A_1	Phenol-Water (72 : 28)	0.43	(+)	(+)	(+)	1	0.43
							(Phosphoryl Choline)
A_2	Metanol-conc. NH_3-Water (60 : 10 : 30)	0.66	(+)	(+)	(−)	1	0.66
A_2	*tert*-Butanol-Picric Acid-Water (80 : 2 : 20)	0.29	(+)	(+)	(/)	1	0.29
A_2	*n*-Butanol-Acetic Acid-Water (4 : 1 : 1)	0.15	(+)	(+)	(−)	1	0.15
A_2	*n*-Butyric Acid-NaOH-Water (69%v/v-0.85%w/v NaOH)	0.71	(+)	(+)	(−)	1	0.71
A_2	Phenol-Water (72 : 28)	0.88	(+)	(+)	(−)	1	0.88

tert-Butanol-Picric Acid-Water; *n*-Butanol-Acetic Acid-Water; Phenol-Water (72 : 28); *n*-Butyric Acid-NaOH-Water; Methanol-Ammonia-Water; Ethanol-28% Ammonia-Water (60 : 30 : 10); Ethanol-28% Ammonia-Water (60 : 35 : 5)

The last two solvents could differentiate A_2 from glycerophosphoryl choline. Thus A_2 was identified as phosphoryl choline.

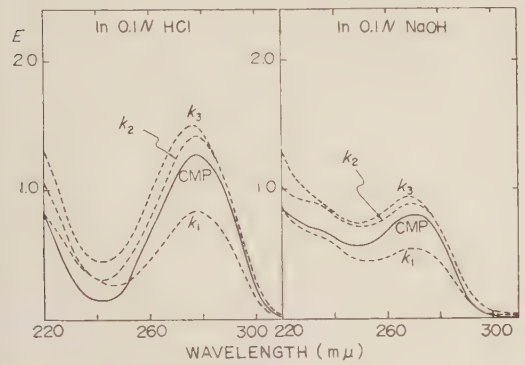

Fig. 4. U.V. Absorption curve of k_1, k_2, and k_3.

Identification of CDP-Ethanolamine and CDP-Choline—As illustrated in Fig. 4, k_1, k_2, and k_3 showed the characteristic U.V. absorption curve of cytosine compound.

Purified k_1 showed positive ninhydrin reaction with both spot test and quantitative metod for amino acid estimation. After hydrolysis with *N* HCl at 100°C for 60 minutes, the hydrolytic products were applied to the Dowex 50 chromatography, and found that k_1 was decomposed into two substances. One was a ninhydrin positive substance having the same eluting position as phosphoryl ethanolamine and the other was a U.V. absorbing substance having the same eluting position as cytidylic acid. These two substances showed the identical R_f values on paper chromatography with phosphoryl ethanolamine and cytidylic acid, respectively. The solvents employed were as follows:

n-Butanol-Acetic Acid-Water; *tert*-Butanol-Picric Acid-Water

Thus k_1 was assumed to be CDP-ethanolamine.

k_2 was first hydrolysed with *N* HCl at 100°C for 30 minutes, and applied to paper chromatography with *n*-butyric acid solvent. As the result, four spots could be found. One corresponded to phosphoryl choline, the second was k_2 remained unhydrolysed, the third was a U.V. absorbing spot and corresponded to cytidylic acid, and the fourth was a small amount of inorganic phosphate. Then k_2 was directly compared with CDP-choline with variety of paper chromatography. The solvents used were as follows:

n-Butanol-Acetic Acid-Water; *n*-Butyric Acid-NaOH-Water; Phenol-Water (72 : 28); *tert*-Butanol-Picric Acid-Water; Methanol-Formic Acid-Water; Methanol-Ammonia-Water; Acetone-Trichloracetic Acid

With all of these solvent systems, k_2 showed the same R_f as CDP-choline. Furthermore, highly purified k_2 was hydrolysed with 6 *N* HCl at 100°C for 60 hours and tested with paper chromatography (*n*-Butanol-Acetic Acid-Water), and choline could be detected on the paper.

Thus k_2 was identified as CDP-choline.

The third U.V. absorbing peak k_3 was identified as cytidylic acid with column chromatography and paper chromatography (Methanol Ammonia-Water; *n*-Butyric Acid-NaOH-Water; Acetone-Trichloracetic Acid). As cytidylic acid could be adsorbed on Dowex 1 formate column, this cytidylic acid in the Dowex 1 formate unadsorbable fraction was considered to be the decomposition product from either k_1 or k_2 or from both.

Another nitrogen-containing phosphoric ester, *o*-phosphoryl serine, was not found in the Dowex 1 formate unadsorbable fraction. Authentic phosphoryl serine could be adsorbed on Dowex 1 formate column and eluted in the next to adenylic acid on the chromatogram. Therefore, the radioactive peak B in liver chromatogram was analysed with paper chromatography. The peak contained at least two ninhydrin positive substances, but both of which differed from phosphoryl serine and had no radioactivity. The main radioactive component of the peak B was

Table III

R_f Values of Nitrogenous Phosphoric Esters

Solvent	P-Ser	P-Eam	P-Ch	k_1 (CDP-Eam)	CDP-Ch
n-Butanol-Acetic Acid-Water (4 : 1 : 1)	0.06	0.12	0.15	0.013	0.020
tert-Butanol-Picric Acid-Water (80 : 2 : 20)	0.27	0.29	0.29	0.014	0.022
n-Butyric Acid-NaOH-Water (69 : 0.85 : 21)	0.38	0.57	0.71	0.52	0.66
Phenol-Water (72 : 28)	0.22	0.43	0.88	0.52	0.88
Acetone-25% Trichlor Acetic Acid (75 : 25)	—	0.69	0.73	0.28	0.35
Methanol-conc. NH_3-Water (60 : 10 : 30)	0.57	0.59	0.66	0.66	0.67

P-Ser: *o*-phosphoryl serine
P-Eam: *o*-phosphoryl ethanolamine
P-Ch: phosphoryl choline
CDP-Eam: cytidine diphosphate ethanolamine
CDP-Ch: cytidine diphosphate choline

negative for ninhydrin spot test.

Table III shows the R_f values of the nitrogen-containing phosphoric esters with variety of solvent systems. k_1 showed similar R_f to phosphoryl etheanolamine with phenol or butyric acid solvent but showed the similar R_f to CDP-choline with alcoholic or acetonic solvent. CDP-choline also showed the similar R_f to phosphoryl choline with phenol or butyric acid solvent but showed far less R_f than phosphoryl choline with alcohol or acetone solvent. This also strongly suggests that k_1 was CDP-ethanolamine. Phosphoryl compounds and CDP-compounds could be resolved with acid-alcohol solvents. Ethanolamine-, and choline-compounds could be resolved with phenol or butric acid solvents. Thus two dimensional paper chromatography with acid-alcohol solvents and polar solvents would be useful for complete separation of the nitrogen-containing phosphoric esters.

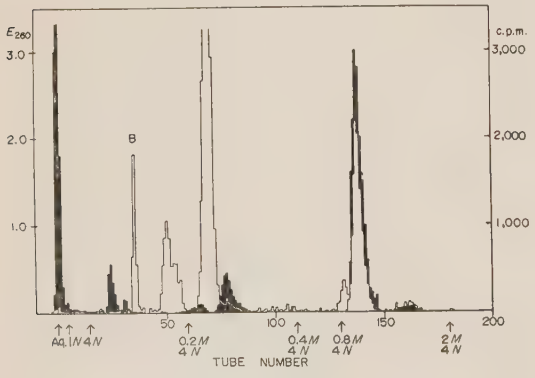

Fig. 5. Chromatography of the acid-soluble extract from rat striated muscle.

One hour after the intraperitoneal injection of P^{32} inorganic phosphate.
rat: 100 g. male; muscle tissue analysed: 1.75 g.
black peaks: U. V. absorption
colorless peaks: radioactivity

Identification of Phosphocreatine on the Dowex 1 Formate Chromatogram—Fig. 5 shows a Dowex 1 formate chromatogram with striated muscle (*2*). The peak B in muscle, which appeared also in the next to adenylic acid was freeze-dried and analysed with paperchromatography. This substance was an extremely labile phosphate and liberated inorganic phosphate spontaneously. After heating the paper at 110°C for 3 hours, a spot could be detected with Jaffe's reaction. This spot showed identical R_f with creatine in the following paper chromatography.

Methanol-Ammonia-Water: *tert*-Butanol Picric Acid-Water; Phenol saturated with Water (acetic acid atmosphere); *n*-Butanol-Acetic Acid-Water; *n*-Butyric Acid-NaOH-Water

Thus the peak B in muscle chromatogram

was assumed to be phosphocreatine.

Chromatography with Authentic Samples—Fig. 6 shows a chromatogram with authentic samples. A good separation could be obtained by this method. When large amounts of salts were present in the sample mixture, a tendency of overlapping of each peak and shifting of the peaks into left side appear. In that case, pretreatment with cation exchanger or rechromatography would be necessary.

Fig. 7 shows a two dimensional paper chromatography of the nitrogen-containing phosphoric esters. *tert*-Butanol-Picric Acid-Water could replace *n*-Butanol-Acetic Acid-Water, and phenol solvent could replace *n*-butyric acid solvent. In any case, the volatile solvent should be applied for the first development.

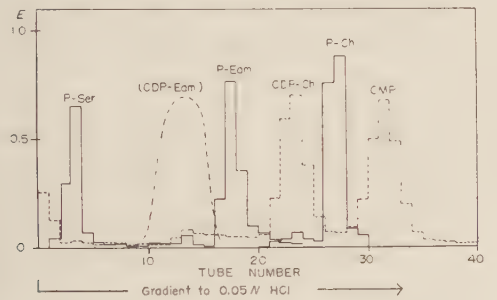

Fig. 6. Chromatography with authentic samples.

column: Dowex 50 (X4, 200—400 mesh, -H form), 0.6×45 cm.

elution: gradient to 0.05 *N* HCl (mixer volume =150 ml., 8—12 ml./hour)

fractions collected: each 4 ml.

approximate amounts of samples contained in the test mixture (2 ml.): CMP, 0.5 mg.; CDP-choline-Na, 0.75 mg.; *o*-phosphoryl ethanolamine-Ba, 2.5 mg.; *o*-phosphoryl serine-Ba, 1.2 mg.; phosphoryl choline-Ca, 2.5 mg.

Barium was removed by pretreatment with amberlite IR 120 in the presence of diluted HCl.

(CDP-Eam) in the figure indicates only the eluting position of CDP-ethanolamine.

Methods of assay: U. V.-absorption at 278 mμ was measured for CDP-choline and CMP. Ninhydrin color value at 570 mμ was measured for phosphoryl serine and phosphoryl ethanolamine (0.5 ml. of the samples were used for the determination). Absorption at 365 mμ of choline periodide in ethylene dichloride was measured for hydrolysed phosphoryl choline (2 ml. of the samples were used).

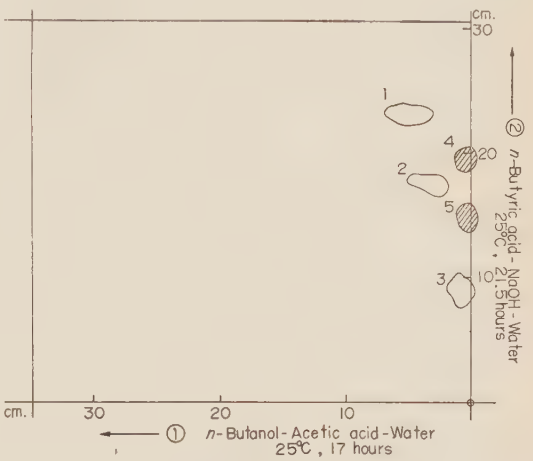

Fig. 7. Paper chromatographic separation of the nitrogen-containing phosphoric esters.

1: phosphoryl choline
2: phosphoryl ethanolamine
3: phosphoryl serine
4: CDP-choline
5: CDP-ethanolamine

DISCUSSION

Concerning the Occurrence and Isolation of Nitrogen-containing Phosphoric Esters—There are several papers in literature concerning the nitrogen-containing phosphoric esters in the acid soluble fraction of animal tissues.

Awapara *et al.* (*13*) reported the presence of free phosphoryl ethanolamine in rat organs and human tumors in 1950. Ansell and Dawson (*14*) also found this substance in the rat brain (1952). Phosphoserine was detected by Malatesta (*15*) in blood, lens of eyes *etc.* in 1952, and by Nemer and Elwyn (*16*) in the rat liver in 1957. The occurrence of an interesting substance, *o*-phosphodiester of L-serine and ethanolamine, in turtle tissue was reported by Roberts and Lowe (*17*) in 1954. CDP-choline was first reported by Kennedy and Weiss (*18*) as an intermediary product of lecithin biosynthesis (1955). Neil (*19*) described the occurrence of phosphryl choline and phosphoryl

ethanolamine in goat placenta (1960). Recently, Baker and Porcellati (*20*) reported the separation of nitrogen-containing phosphate esters from brain and spinal cord with ion-exchange chromatography (1959). They used Zeo-Karb bufferized with citrate and separated phosphoryl serine, phosphoryl ethanolamine, phosphoryl choline, glycerophosphoryl choline, and glycerophosphryl ethanolamine.

The isolation method described in this paper will provide some additionals to these findings. Furthermore, the simultaneous isolation of phosphoryl-compounds and CDP-compounds in relatively pure state and in a simple way will give a powerful crue for the metabolic studies of these esters.

Concerning the P^{32}-Incorporatin into the Nitrogen-containing Phoshoric Esters—As phosphoryl ethanolamine and phosphoryl choline are known to be the intermediary metabolites of lipid synthesis, it seems to be reasonable that these phosphate showed high radioactivity in the metabolically active or reproductive tissues. However, it was an unexpected observation that the next-comming intermediary metabolite, CDP-ethanolamine and CDP-choline, had practically no radioactivity one hour after the injection of P^{32}. On the basis of the ninhydrin color value, the amount of CDP-ethanolamine in liver was assumed to be less than one tenth of the amount of phosphoryl ethanolamine. But, even with this small pool amount of CDP-compounds in the tissue, some radioctivity could be expected to present if they all pass through the known metabolic pathway.

Phosphoryl ethanolamine was reported to be derived from phosphoryl serine by Nishizawa *et al.* (*21*) in 1958. Choline phosphokinase is known to present in many animal tissues and purified from yeast (*22*). But, as to ethanolamine, the direct phosphorylation mechanism is not clear yet, although some assumption has been made.

In any way, the actual mechanisms of the P^{32}-incorporation *in vivo* into the nitrogen-containing phosphates are desired to be investigated.

SUMMARY

1. The acid-soluble fraction from tissues of rats injected with P^{32} were treated with Dowex 1 formate and the unadsorbable fraction was further analysed by the column chromatography with Dowex 50.

2. Radioactive *o*-phosphoryl ethanolamine and *o*-phosphoryl choline were isolated and identified on the Dowex 50 chromatogram.

3. CDP-ethanolamine and CDP-choline showing practically no radioactivity were also isolated and identified on the Dowex 50 chromatogram.

4. Phosphocreatine in muscle extract was identified on the Dowex 1 formate chromatogram.

5. Both column- and paper-chromatographic method for the separation of nitrogen-containing phosphoric esters were described.

The author expresses heartful thanks to Prof. N. Shimazono for his kind advice and encouragement.

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Inhibitory Effect of Light on Oxygen-Uptake by Cell-Free Extracts and Particulate Fractions of *Rhodopseudomonas palustris*

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In 1956, Morita (*1*) reported that the oxidative metabolism of *Rhodopseudomonas palustris*, a non-sulfur purple bacterium, is subject to profound changes on illumination. He observed that when lactate and pyruvate were used as substrate, the oxygen-uptake of the resting cells was markedly suppressed in the light. In the present work, the study was extended to various other substrates that serve as substraies in the oxidative metabolism of the organism, and the same effect of light was found to occur with all the substrates tested. It was demonstrated with cell-free extracts of bacterial cells that the oxidative capacity, which was found to be located in the particulate fractions, was also suppressed by illumination. The experimental results are briefly described in the following.

MATERIAL AND METHODS

Rhodopseudomonas palustris, isolated by Morita in this laboratory, was used throughout this work. The culture conditions were the same as previously described (*1*), with the minor modification of adding 0.25% ammonium sulfate to the medium.

Preparation and Fractionation of Cell-Free Extract—The washed cells usually harvested from two-day cultures, were ground with an equal weight of quartz sand in a mortar for 40 minutes and then extracted with three volumes of ice-cold sucrose (0.2 *M*)-phosphate (0.01 *M*) mixture (pH 7.4). In some experiments, cells suspended in the same mixture were disrupted by applying sonic vibration, using a 10 kc Toyo Sonic Oscillator for 5 minutes at temperature below 5°C. The results obtained by these methods were almost identical.

After grinding or sonicating the cells, the mixture obtained was centrifuged at $10,000 \times g$ for 20 minutes to remove intact cells and larger cell fragments, and the supernatant was further centrifuged at $24,600 \times g$ for 60 minutes. The sendiment consisted of dark red particles which seemed to correspond to the "chromatophore" isolated by Schachman *et al.* (*2*) from *Rhodospirillum rubrum*, using a similar procedure. The supernatant obtained was still redish in color, probably containing chromatophore fragments. This fraction will hereafter be called the "colored supernatant".

Assay Procedures—The oxidative activity of these fractions was measured at 25°C in a Warburg manometer in a dark room. The activity was expressed as the amount of oxygen absorbed per mg. dry weight or mg. protein (determined by the biuret reaction) for one hour, during which time a constant reaction rate was usually found to be maintained. The reaction mixture, 3.0 ml. in total volume, consisted of 100 μmoles of phosphate buffer (pH 7.4), 50 μmoles of the substrate (pH adjusted to 7.4 with sodium hydroxide), and the material to be tested. Each vessel usually contained 20 mg. dry weight of intact cells or 0.5–3.0 mg. protein of cell-free preparation. In the centre well of the vessel was placed 0.3 ml. of 15% potassium hydroxide solution. A bank of five 100-Watt incandescent lamps, placed under the glass bottom of the thermostat, was used for the illumination, which was 3,200 lux at the level of the reaction vessels.

The dehydrogenase activity was determined by measuring spectrophotometrically the reduction of 2,6-dichlorophenol indophenol; the reaction mixture consisted of 100 μmoles of phosphate buffer (pH 7.4), 50 μmoles of substrate, 0.1 μmoles of the dye and 0.5 ml. of the extract (*ca.* 0.5 mg. protein); total volume 3 ml. After 5 minutes of incubation in a water bath at 25°C in the dark or in the light (two 100-watt incandescent lamps; about 2,000 lux), the decrease in optical density at 600 mμ was measured. To avoid reoxidation of the reduced dye in the light (*3*), the reaction was carried out in a cell attached to a Thunberg tube, which was thoroughly evacuated and flushed three times, and finally filled, with oxygen-free nitrogen.

RESULTS

Effect of Light on Oxygen-Uptake by Cells—The following substances were found to be utilized as the substrates of respiration by intact cells of *Rhodopseudomonas palustris:* α-ketoglutarate, succinate, fumarate, lactate, pyruvate, acetate, propionate, glutamate and methionine (Table I). Citrate was not utilized by the intact cells, probably owing to the permeability barrier, since the acid was readily oxidized by the crude cell-free extract (cf. Table II).

TABLE I

Effect of Light on Oxygen-Uptake by Intact Cells

Composition of reaction mixture in text. Results expressed as μl. oxygen absorbed per mg. (dry weight) of cells in 60 minutes.

Substrate	Oxygen-uptake		L/D
	Dark (D)	Light (L)	
—	4.5	2.2	0.49
Citrate	4.4	2.1	0.48
α-Ketoglutarate	9.7	2.3	0.24
Succinate	7.9	3.2	0.41
Fumarate	7.7	3.6	0.47
Malate	12.5	6.1	0.49
L-Glutamate	12.8	10.4	0.81
DL-Lactate	13.5	5.7	0.42
Pyruvate	5.4	3.8	0.70
Acetate	9.6	3.9	0.41
Propionate	11.9	2.8	0.24

As will be seen in Table I, the photo-inhibition of the respiratory oxygen-uptake was observed with all the substrates utilized, and also in the case of the endogenous respiration without added substrate. Let us denote the extent of photo-inhibition of oxygen-uptake by the ratio L/D——L and D representing the rates of oxygen-uptake in light and dark. The ratio was found to fluctuate from one preparation to another within a range from 0.20 to 0.85. However, this light-effect was almost unaffected by various treatments; neither storage of the resting cells at 3°C for a month, nor lyophilization of the cells, abolished the photo-inhibitory effect. On exposing the cells to temperature over 35°C for 24 hours, the capacity for oxidizing most of the tested substrates was found to be abolished, although the endogenous respiration as well as its photo-sensitivity remained intact even after such treatment.

Effect of Light on Oxygen-Uptake by Cell-Free Extract—The cell-free extract of the bacterium was found to retain the oxidative activities towards most of the acids in the tricarboxylic acid cycle, including citrate. Glutamate, lactate, pyruvate, acetate, propionate and methionine were not oxidized with this extract, in the sense that the addition of these acids did not increase the ratio of oxygen-uptake over the endogenous level.

TABLE II

Effect of Light on Oxygen-Uptake by Crude Cell-Free Extract

Composition of reaction mixture in text. Results expressed as μl. oxygen absorbed per mg. protein in 60 minutes.

Substrate	Oxygen-uptake		L/D
	Dark (D)	Light (L)	
—	6.0	3.3	0.55
Citrate	15.5	12.2	0.79
α-Ketoglutarate	12.3	10.2	0.83
Succinate	12.8	10.9	0.85
Fumarate	10.8	8.7	0.81
Malate	9.6	5.8	0.60

Oxidative activity of the extract varied, from one preparation to another, with most of the acid utilized, while an almost constant level of oxidation was always obtained with succinate as substrate. To obtain a maximum rate of oxidation in the presence of succinate, a phosphate concentration over 1/100 *M* in the reaction mixture was required. Therefore, a phosphate buffer of 1/30 *M* (pH 7.4) was always employed in these experiments. The activity-pH curves for the oxidative reaction, in light and dark, with succinate as substrate, are shown in Figure 1. Tris-

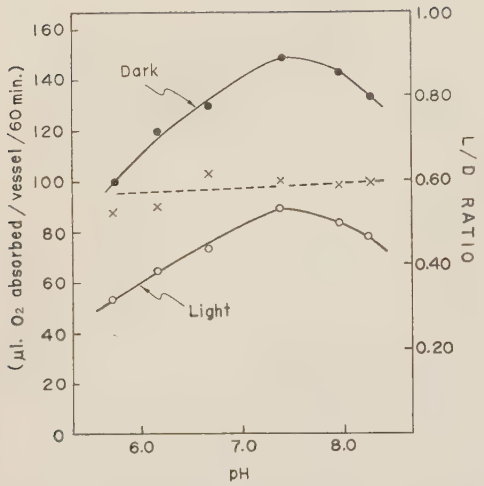

FIG. 1. Oxidation of succinate by cell-free extract of *Rhodopseudomanas palustris* as influenced by pH.

Reaction medium as in text. —●— oxygen-uptake in the dark, —○— oxygen-uptake in the light, —×— L/D ratio.

(hydroxymethylaminomethane)-buffer did not replace the phosphate buffer. Glycylglycine buffer was found to have a considerable inhibitory effect.

Now, the oxygen-uptake of cell-free

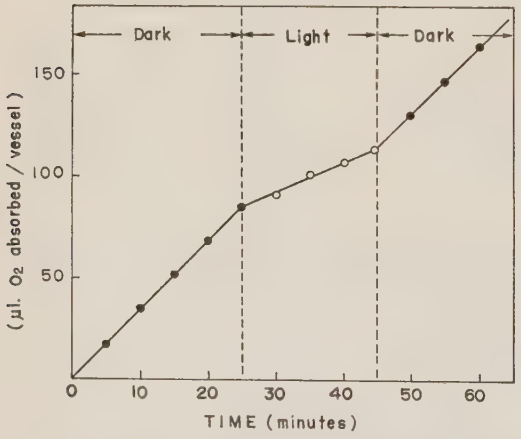

FIG. 2. Time course of oyxgen-uptake of cell-free extract of *Rhodopseudomonas palustris* under alternating dark and light conditions.

preparations was also found to be suppressed by illumination. The magnitude of the photo-inhibition was usually smaller with cell-free extract (L/D about 0.5–0.9) than with intact cells, irrespective of the substrate added and pH of the reaction medium. Figure 2 shows the time-course of oxygen-uptake of a cell-free extract under alternating dark and light conditions. The photo-inhibition observed with this preparation was, like that with intact cells, found to be completely removed when the illumination was stopped. Reillumination immediately restored the previous level of suppression.

Effect of Light on Oxygen-Uptake by Various Fractions—The distribution of oxidative activities among various fractions obtained by fractional centrifugation of the crude cell-free extracts, as well as their photo-sensitivity, was investigated. The results obtained with chromatophores and colored supernatant are summarized in Table III. It will be seen

TABLE III

Effect of Light on Oxygen-Uptake by Chromatophores and Colored Supernatant

Composition of reaction mixture in text. Results expressed as μl. oxygen absorbed per mg. protein in 60 minutes.

Substrate	Oxygen-uptake by					
	Chromatophore			Colored supernatant		
	Dark	Light	L/D	Dark	Light	L/D
—	0.6	0.3	0.50	2.0	1.6	0.80
Citrate	30.6	13.6	0.45	12.1	10.8	0.89
α-Keto-glutarate	26.1	12.3	0.47	3.6	3.0	0.83
Succinate	30.8	6.2	0.20	11.2	8.0	0.71
Fumarate	9.3	3.4	0.37	6.6	4.8	0.73
Malate	9.3	1.1	0.12	6.3	4.2	0.67

that all the acids utilized by the crude extract are also oxidized by both fractions. In this case, also, a rapid and steady rate of oxidation was consistently obtained with succinate, whereas oxidation of other acids was subject to considerable fluctuation. As will be seen in Table III, the oxygen-uptake of chromatophores and supernatant fractions was suppressed by illumination, L/D ratio being 0.1–0.9 with the former and 0.6–0.9 with the latter. The finding that the oxida-

tion of colored supernatant fraction was also inhibited by illumination, suggests a close association of the oxidative system with the photo-active pigment system in this fraction.

The colored supernatant was, then, further fractionated by centrifugation at 60,000 $\times g$ for 60 minutes into a small-particulate fraction of red color and a pigment-free supernatant. Oxidative activity for succinate was found only in the former fraction (Table IV). Illumination also inhibited the oxygen-uptake of small particles, to a degree comparable with that of chromatophores. Table IV also includes the results of an experiment, in which the succinate oxidase activity was followed, in dark and light, with the chromatophore fragments obtained by disrupting the isolated chromatophores in a sonic oscillator at 10 kc for 10 minutes and then by centrifuging at 24,000 $\times g$ for 60 minutes. It was found that the oxidative activity as its light-sensitivity in chromatophore fragments were apparently similar to those in the small particles. This suggests that the observed activities in the colored supernatant is due to the disrupted chromatophore.

TABLE IV

Effect of Light on Oxygen-Uptake by Various Cell-Fractions

Composition of reaction mixture in text. Results expressed as μl. oxygen absorbed per mg. protein in 60 minutes.

Fraction	Oxygen-uptake		L/D
	Dark (D)	Light (L)	
Colored supernatants	9.7	5.1	0.53
small particles	13.9	6.8	0.49
coloreless supernatant	0	0	—
Chromatophores	26.4	10.1	0.38
fragments obtained by sonic disruption	15.6	5.0	0.32

Effect of Light on Anaerobic Dehydrogenation—The effect of light on the dehydrogenase activity of the cell-free extracts was investigated, using succinate and lactate as substrates. The decolorization of the acceptor, 2,6-dichlorophenol indophenol was followed spectrophotometrically at 600 mμ. The dye was used in a concentration of 3.3×10^{-5} M which showed no significant effect on the oxidative activity or its photo-sensitivity. In striking contrast to the results obtained in aerobic experiments, neither inhibition nor acceleration of the reaction was caused by illumination in these anaerobic experiments (Table V).

TABLE V

Effect of Light on Dehydrogenase Activities

Composition of reaction mixture in text.

Substrate	Decrease in optical density at 600 mμ		L/D
	Dark (D)	Light (L)	
—	0.029	0.020	(0.69)
Succinate	0.285	0.285	1.00
Succinate	0.301	0.291	0.94
—	0.017	0.022	(1.28)
Lactate	0.163	0.169	1.04

The aerobic oxidation of reduced cytochrome c, ascorbic acid, hydroquinone and *p*-phenylenediamine was also investigated. The oxygen-uptake with the three latter substrates, as well as the oxidation of reduced cytochrome, was found to be markedly accelerated by illumination; a result in accord with the previous findings on photo-oxidation (*3*).

Effect of Inhibitors and Heat Treatment—The effect of various inhibitor and heat treatment on the photo-sensitivity of oxygen-uptake was investigated, using the cell-free extracts. The results obtained are summarized in Table VI. The succinoxidase activity of the extract was more or less inhibited by the following inhibitors; heavy metal poisons (cyanide, azide and hydroxylamine), sulfhydryl poisons (*p*-chloromercuribenzoate and arsenite), uncouplers (2,4-dinitrophenol and arsenate), a dye (2,6-dichlorophenol indophenol) and malonate. None of them, however, specifically affected the photo-inhibition in question. Antimycin A and *o*-phenanthroline did not affect neither oxidation nor photo-sensitivity. Adenosine diphos-

TABLE VI

Effect of Inhibitors and Heat Treatment on Oxidative Activity of Crude Cell-Free Extract

Composition of reaction mixture in text. Succinate as substrate.

Inhibitor or Treatment	(M)	μl. oxygen absorbed per vessel in 60 minutes		L/D
		Dark (D)	Light (L)	
Cyanide	control	98.3	75.5	0.77
	$1\cdot10^{-4}$	60.1	44.3	0.74
	$1\cdot10^{-3}$	14.8	11.7	0.86
Azide	control	85.8	68.3	0.80
	$3\cdot10^{-4}$	72.7	56.9	0.78
	$1\cdot10^{-3}$	67.7	59.9	0.88
Hydroxylamine	control	97.5	63.6	0.65
	$1\cdot10^{-4}$	78.3	41.8	0.54
	$1\cdot10^{-3}$	63.6	34.8	0.55
Antimycine A	control	43.6	30.5	0.70
	100γ/vessel	47.0	30.5	0.65
Malonate	control	97.5	63.6	0.65
	$5\cdot10^{-3}$	47.8	20.9	0.44
p-Mercuribenzoate	control	87.7	46.1	0.53
	$3\cdot10^{-5}$	56.1	19.3	0.34
Arsenite	control	70.1	56.9	0.81
	$6\cdot10^{-4}$	62.2	50.8	0.82
Arsenate	control	50.5	15.7	0.31
	$2\cdot10^{-3}$	40.1	10.4	0.26
	$8\cdot10^{-3}$	34.0	11.3	0.33
2, 4-Dinitrophenol	control	70.1	56.9	0.81
	$3\cdot10^{-4}$	68.3	61.3	0.90
2, 6-Dichlorophenol indophenol	control	79.7	62.3	0.78
	$1\cdot10^{-3}$	53.1	33.0	0.62
o-Phenanthroline	control	95.7	63.6	0.67
	$1\cdot10^{-3}$	96.4	63.6	0.66
Adenosine diphosphate	control	142.8	119.5	0.84
	$1.25\cdot10^{-3}$	64.6	58.6	0.91
Heat treatment for 5 minutes	control	61.3	50.8	0.83
	40°C	46.9	46.9	1.00
	60°C	10.5	10.5	1.00
	100°C	0	0	—
Freezing-thawing	control	99.3	53.2	0.54
	treated	128.8	73.5	0.57

phate (1.25×10^{-3} M) was found to induce a significant inhibition of oxidation both in dark and light, without causing any change in the values of L/D ratio. A remarkable stimulation of oxygen-uptake was observed on freezing-thawing but the L/D ratio remained constant. Only on heat treatment did the L/D ratio increase, finally reaching unity. This indicates that the mechanism of photo-inhibition is sensitive to high temperature.

DISCUSSION

It is a well known fact that non-sulfur purple bacteria, such as *Rhodopseudomonas* and *Rhodospirillum*, are capable of growing on synthetic media containing various organic compounds. In the light, growth takes place either aerobically or anaerobically; while in the dark, growth is possible only under aerobic conditions. It is evident that the energy necessary for the bacterial growth is provided by light under light-anaerobic condition and by the respiratory metabolism under the dark-aerobic condition. The changes in the aerobic metabolism induced by illumination are of interest in elucidating the links between the two types of metabolism. The fact that light causes suppression of oxygen-uptake, accompanied, on the other hand, by a marked acceleration of the assimilatory processes of the cells, shows that the energy-yielding process is switched by the light from the oxidative to the photochemical metabolism.

The present work has revealed that the chromatophores in this bacterium possess the capacity to carry out the complete oxidation of various substrates with molecular oxygen as final electron acceptor. Oxidation observed with the "colored supernatant" is also due to the activity of small particles which were probably identical with fragments obtained from isolated chromatophores by sonic disruption. It was also found that oxidative activities of these particulate fractions, like that of the intact cells, underwent a rapid and reversible inhibition on illumination. These findings suggest that the switching effect of light on the oxidative metabolism occurs primarily in the chromatophores. In

this connection, it is interesting that the illuminated chromatophores or their fragments show a capacity to catalyze the esterification of inorganic phosphate in the presence of phosphate acceptor (*4*). Thus, it may be inferred that photophosphorylation supplies energy sufficient to maintain a high level of assimilative activity of the bacterium even under a suppressed state of its respiratory activity in the light.

On the other hand, the possibility that this photophosphorylation plays a role in the actual mechanism of the photo-inhibition in question by competing a common phosphate acceptor with oxidative phosphorylation is ruled out by the following reasons: 1) photo-inhibition of oxygen-uptake of the cell-free preparation is not removed by the addition of phosphate acceptor (adenosine diphosphate) or uncouplers. 2) The addition of *o*-phenanthroline and 2,6-dichlorophenol indophenol does not affect this photo-inhibition, in concentrations which have been shown to be effective in blocking the photochmical phosphorylation of *R. rubrum*-extract (*4*). 3) The bacterial extract, which showed a suppressive rate of oxidation in light, was found to catalyze the oxidative phosphorylation, but only at a rate insufficient to explain the above effect: the ratio of mole of phosphate esterified per atom of oxygen absorbed, with succinate as electron-donor, amounted to only 0.2–0.5.*.

Recently, White and Vernon have reported that light-aerobic incubation of the chromatophores of *R. rubrum* resulted in an inhibition of the DPNH-oxidase activity in this fraction and inferred that the photo-inhibition occurs at a step in which flavoprotein is involved (*5*). This phenomenon, however, seems to be different from the photo-inhibition observed in the present work on intact cells and cell-free preparations, since photo-inhibition of DPNH-oxidase has been reported to be a progressive reaction, reversed only slowly after a significantly long period of dark incubation (*cf.* Fig. 2).

* unpublished data.

The capacity of the bacterial extract to carry out photooxidation of reduced cytochrome c in light, first described by Kamen and Vernon (*3*), can in no way participate in the mechanism of photo-inhibition, since this photooxidation requires molecular oxygen as final electron acceptor. Moreover, the above-mentioned heat-sensitivity of the photo-inhibition is also incompatible with the thermostability of the aerobic photooxidation of reduced cytochrome. The fact that illumination, suppressing the oxygen-uptake of the bacterial extract with succinate as substrate, on the one hand, stimulates the same process with reduced cytochrome c or dye as hydrogen donor, indicates that the electrons derived from the former substrate are transported through a "closed" respiratory chain in the particles, *i.e.*, all the components of the oxidative system are inferred to undergo alternate oxidation-reduction without electron exchange with cytochrome c and dyes added to the reaction medium. If this were not so, photooxidation of the substrate mediated by the added cytochrome or dye would mask the light-induced inhibition.

On the other hand, there is a possibility that the photo-inhibition under investigation may be caused by a change in the oxidation-reduction level in one or another link in the electron transport chain**, or more precisely, the production of an oxidizing substance which will compete with molecular oxygen. In fact, such oxidative-change in cytochrome system has been observed on illuminating intact cells (Duysens (*6*), Chance and Smith (*7*) and Chance and Nishimura (*8*)) as well as chromatophores (Vernon (*9*)) under anaerobic conditions. The results of the anaerobic experiments in the present study indicate that the site of the suggested change, if it occurs, may be a step nearer to oxygen than to the site of dehydrogenation.

** In his theoretical consideration on the kinetics of a consecutive oxidation-reduction reaction, Takamiya has pointed out the possibility that a change in stationary level of an intermediary electron carrier occurring in the reaction chain may cause a blockage of the overall reaction (*10*).

SUMMARY

1. The inhibitory effect of light on the respiratory oxygen-uptake of a non-sulfur purple bacterium, *Rhodoseudomonas palustris* was investigated.

2. The exygen-uptake of intact cells in the presence of various substrates was found to be suppressed by illumination.

3. The cell-free extract of the bacterium retains the capacity to oxidize the acids of tricarboxylic acid cycle. An inhibitory effect of light on the oxygen-uptake was also observed with this fraction.

4. The cell-free extract was fractionated into chromatophore and colored supernatant fractions, and the latter into small particles and colorless supernatant. It was found that the first three (colored) fractions possess the capacity for oxidation of the above-mentioned substrates, while the colorless fraction was essentially inactive. Light inhibition of the oxygen-uptake was observed with all these colored fractions.

5. Light has no effect on the rate of anaerobic dehydrogenation of succinate and lactate with 2, 6-dichlorophenol indophenol as hydrogen acceptor.

6. The effect of various inhibitors and heat treatment on the rate of succinate oxidation in dark and light was investigated.

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Studies on Isokinetin and its Analogs

I. Synthesis of Isokinetin, 2-N-Furfurylaminopurine, and its Activity of Promoting Growth of Leaves

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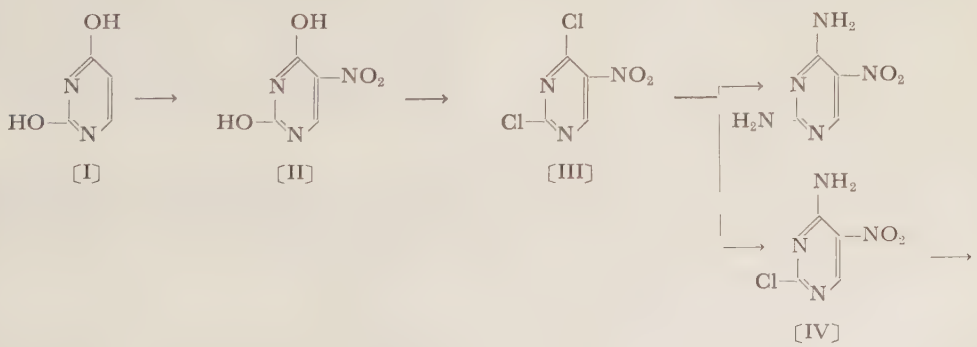
Kinetin, a cell division promoting factor, has been isolated from aged or autoclaved DNA and its chemical structure has been confirmed as 6-(N-furfuryl)-aminopurine (*1*) by synthesis.

Recently Kuraishi and Okumura (*2*) discovered another interesting physiological activity of kinetin, *i.e.*, its stimulating effect upon the growth of green leaves. When disks of *Raphanus* leaf (grown in the open) were kept floating on solutions of kinetin of appropriate concentrations, there occurred more or less remarkable increase in the area of disks. With the kinetin solution of 1 mg./liter for example, which was found to be the optimum concentration for the said effect, the increase in the leaf-area attained to as much as 70 per cent after 20 hours of incubation. This increase was found to be due to the increase of cell volume rather than to that of cell number. It was thus revealed that kinetin has a promoting action upon the growth of leaves, which has never been reported for other growth factors.

One of the authors, Okumura, has been persuing the synthesis of kinetin-analogs which belong to 6-(N-substituted)-aminopurine to investigate the relationships between the chemical structure and the leaf-growth promoting activity (*3*). During the course of this study, our interest was directed to the question as to whether the leaf-growth promoting activity still remains or disappears if the furfurylamino group at the 6-position of the purine ring migrates to the 2-position. After many unfruitful trials, we succeeded in synthesising 2-(N-furfuryl)-aminopurine through the process outlined in Fig. 1.

Nitration of uracil with fuming nitric acid (1.5 D) gave 2,6-dihydroxy-5-nitropyrimidine (II) with 90.7 per cent yield; m.p. 294~295°C. This was chlorinated by phosphoroxychloride in the presence of dimethylaniline to obtain 2,6-dichloro-5-nitropyrimi-



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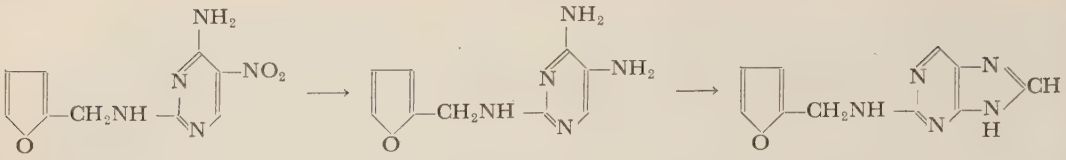


Fig. 1. Synthesis of isokinetin, 2-(N-furfuryl)-aminopurine.

dine (III) with 64 per cent yield.

Amination of (III) by ammonia in methanol-ether solution gave a mixture of 2-chloro-6-amino-5-nitropyrimidine (IV) and 2,6-diamino-5-nitropyrimidine. However, 2-chloro-6-amino-5-nitropyrimidine (IV) could be obtained with 60 per cent yield when amination took place at low temperatures (below zero degree). (IV) was then converted into 2-furfurylamino-6-amino-5-nitropyrimidine (V) with 69.7 per cent yield by refluxing the latter with furfurylamine for 2~4 hours.

2-Furfurylamino-6-amino-5-nitropyrimidine was finally reduced catalytically with Raney-Nickel in methanol to give 2-furfurylamino-4,5-diaminopyrimidine (VI) which was refluxed with formamide to form 2-(N-furfuryl)-aminopurine (VII), the yield being 34.2 per cent. Tan colored needles obtained from absolute alcohol showed a m.p. of 195~196°C. The ultra-violet spectrum of this compound (VII), isokinetin, showed almost the same absorption as that of kinetin.

According to the method of Takematsu (*4*), using *Raphanus* cotyledon as meterial, the leaf-growth promoting activity of isokinetin was compared with that of kinetin.

EXPERIMENTALS

2,6-Dihydrox-5-nitropyrimidine (*II*)—To 8 ml. of fuming nitric acid 4 g. of uracil was added in small portions under stirring at 62~66°C. After continuing stirring for 1.5 hours until the crystal appeared, the temperature was raised up to 70°C to evaporate the excess of nitric acid. To the residue, a small amount of ice-water was added and stirred. When the product was filtered, washed with water, and recrystallized from water, it gave colorless needles, m.p. 294~295°C (92.5 per cent yield).

Anal. Found: N, 26.70%

Calcd. for $C_4H_3N_3O_4$: N, 26.70%

2,6-Dichloro-5-nitropyrimidine (*III*)—A mixture of 10.7 g. of (II), 54 ml. of phosphoroxychloride and 13 ml. of dimethylaniline was refluxed at 120~130°C for 1.5 hours. During this period, the color of the reaction mixture turned from yellowish brown to dark green. After distilling off the excess of phosphoroxychloride, the reaction mixture was poured into ice water under stirring. The residual oily substance was taken up in ether and washed with water. The crude oily product of (III) was obtained after removal the ether by distillation. Yield of crude product was 8.5 g.

2-Chloro-6-amino-5-nitropyrimidine (*IV*)—To a solution of 8.5 g. of (III) in 60 ml. of ether, 10 ml. of methanol saturated with ammonia was added dropwise at 0~5°C under stirring. The mixture was stirred for 30 minutes at the same temperature. After completing the reaction, the excess ammonia was neutralised carefully with 2*N* methanolic hydrochloric acid under cooling. The product was filtered and washed with water. Recrystallization from the mixture of methanol and water (2:1) gave orange colored needles, m.p. 220~221°C (yield 60 per cent).

Anal. Found: N, 31.95%,

Calcd. for $C_4H_3N_4O_2Cl$: N, 32.10%

2-Furfurylamino-6-amino-5-nitropyrimidine (*V*)—A mixture of 1.0 g. of furfurylamine and 1.0 g. of (IV) was refluxed for 1.5 hours at 90~105°C. The product was allowed to stand to crystallized, then filtered and washed. Recrystallization from absolute alcohol gave tan colored, prisms, m.p. 159~160°C with 67 per cent yield.

Anal. Found: N, 29.50%,

Calcd. for $C_9H_9N_5O_3$: N, 29.76%

2-(N-Furfuryl)-Aminopurine (*VII*)—To 15 ml. of methanol suspension containing 0.4 g. of (V) was added 0.6 g. of Raney-Nickel and the mixture was shaken in a hydrogen atmosphere for 2 hours to absorb the calculated amount of hydrogen. The catalyst was then filtered off, the filtrate concentrated to a small volume under reduced pressure to obtain 0.2 g. of crude 2-furfurylamino-5,6-diaminopyrimidine (VI).

A mixture of 0.6 g. of (VI) and 10 ml. of formamide was refluexed at 135~145°C for 2 hours, and excess formamide was removed under reduced pressure. The residue was allowed to stand to crystallize, and then washed well with water. Recrystallization from hot water gave redish-orange needles in a yield of 24 %, m. p. 195~196°C.

Anal. Found: C 55.88, H 4.01, N 32.55%,

Calcd. for $C_{10}H_9N_5O$: C 55.55, H 4.67, N 32.37%

Inhibitory Effect of Kinetin and Isokinetin upon the Growth of Roots of Raphanus sativa the Brassica juncea— Ten ml. each of the culture solutions, to which each 500 mg., 100 mg., 10 mg., and 5 mg./liter of kinetin or isokinetin were added, were dispensed in a Petri dish in which a filter paper had been placed. Each dish was sown with well selected seeds of *Raphanus sativa* or *Brassica juncea* and after incubation lasting for 5 days at 18°C, observations were made of the inhibitory effects of kinetin and isokinetin upon the growth of roots.

RESULTS

Raphanus seeds were allowed to germinate by being incubated on sand at 15-18°C, and on the 4th day of their germination, both sides of cotyledons were spraying with a 200 mg./liter solution of kinetin or isokinetin by using a 0.4 ml.-micro-sprayer. The increase of the leaf-area was measured 5 days after spraying. As is seen from the results shown in Fig. 2, isokinetin had a stimulatory effect on the leaf-growth, in its effect of 80 per cent to that of kinetin (see Fig. 3.).

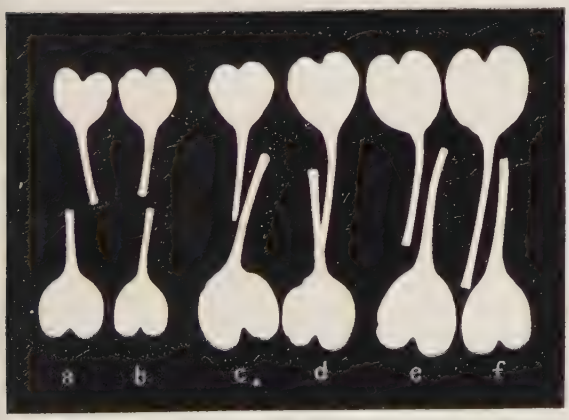


Fig. 2. Effects of kinetin and isokinetin on cotyledons of *Raphanus*.
a, b, : control. c. d, : with isokinetin.
e, f, : with kinetin.

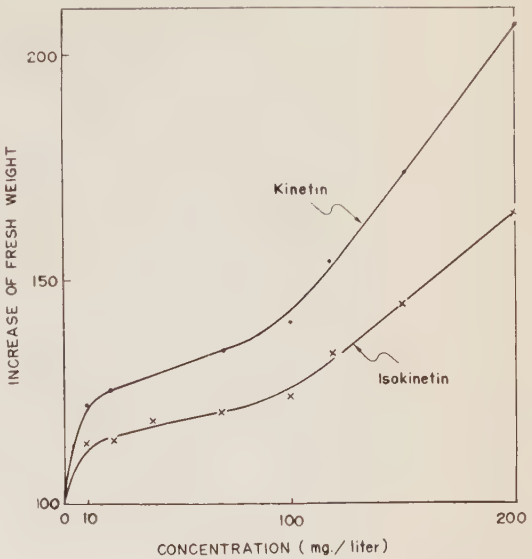


Fig. 3. Increase in fresh weight of *Raphanus* cotyledon caused by spraying of kinetin and isokinetin solution of various concentrations.

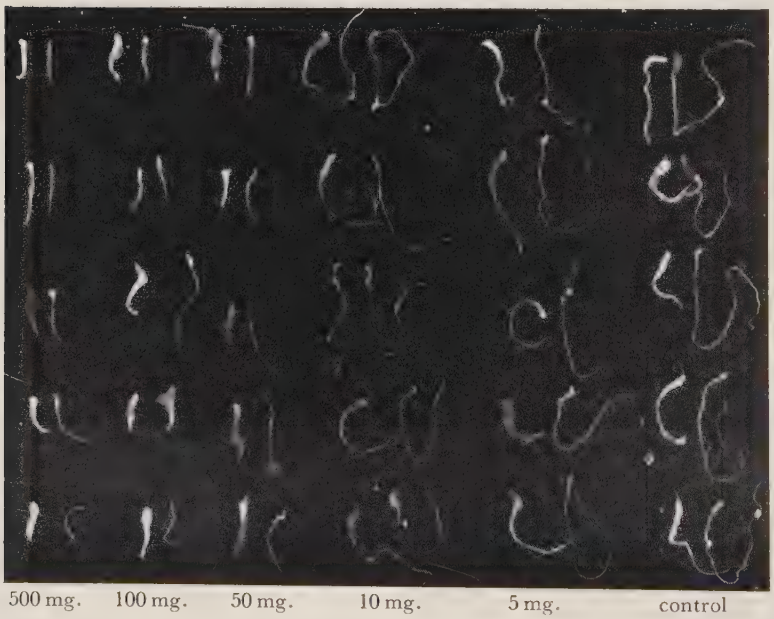
Kuraishi and Okumura* observed that kinetin inhibits the growth of the root of *Brassica chinensis* J. var. *amplexicaulis* Makino at the concentration of 10^{-3} mg./liter. In the present paper we observed the same inhibitory effect of both kinetin and isokinetin and isokinetin upon the growth of roots of *Raphanus sativa* and *Brassica juncea*. The results are shown in Figs. 4 and 5.

As may be seen from Figs. 4 and 5. kinetin inhibited the growth of roots remarkably, even in the concentration of 5 mg./liter, whereas the inhibitory effect of isokenetin was obserbed only at the concentration of 50 mg./liter or higher. It should be pointed out in this connection that gibberelline and indole acetic acid have no stimulating effect on the growth of green-leaf and have no inhibitory effect upon the growth of root. The inhibitory action described above may, therefore, be regarded as being specific for the group of kinetin and isokinetin.

* Okumura F. S., and Kuraishi, S., Uupublished work.

500 mg. 100 mg. 50 mg. 10 mg. 5 mg. control

Fig. 4. Inhibitory effect of kinetin upon the growth of roots of *Raphanus* and *Brassica*. In each pair, *Raphanus* is on the left, and *Brassica* is on the right.



500 mg. 100 mg. 50 mg. 10 mg. 5 mg. control

Fig. 5. Inhibitory effect of isokinetin upon the growth of roots of *Raphanus* and *Brassica*. In each pair, *Raphanus* is on the left, and *Brassica* is on the right.

SUMMARY

1. Isokinetin, 2-(N-furfuryl)-aminopurine, was synthesised and its effect of promoting growth of leaves was compared with that of kinetin, using *Raphanus* and *Brassica* as materials.

2. Isokinetin stimulates the growth of leaf, the activity being however, about 80 per cent of that of kinetin.

3. Both kinetin and isokinetin inhibit the growth of roots of *Raphanus sativa* and *Brassica juncea*.

4. Emphasis was laid on the fact that the mode of action of kinetin and isokinetin is profoundly different from those of gibberelline and indole acetic acid which are known to have no activity of stimulating growth of leaves and inhibiting growth of of roots.

The authors wish to express their hearty thanks to Prof. M. Kuru, School of medicine, Osaka University and Prof. R. Kinoshita, City of Hope Medical Institute, Duarte, California, U.S.A., for their interests in this work and encouragements throughout this research. They are also very grateful for a grant from the ministry of Education.

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A Labile Phosphorus Compound Showing Phosphorus Turnover Appearing in Protein Fraction of A Mycobacterium

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In the course of P^{32}-incorporation studies in a *Mycobacterium* the author found that a labile phosphorus fraction appeared transiently in the protein fraction obtained by the Schneider procedure (*1*). This phenomenon is described in this paper.

MATERIALS AND METHODS

An avirulent strain of *Mycobacterium avium*, strain Jucho, was used throughout. This organism was considered to be favorable for the study because of its slow growth, which made it easier to follow the course of P^{32}-incorporation. Seven-day-old cultures grown on Sauton medium were used. At this time of incubation the culture was known to be at the stationary growth phase (*2*). The cells were washed in saline three times and suspended in phosphorus-deficient medium containing 1.5 μc/ml. P^{32}-orthophosphate (The Radiochemical Center, Amersham, England) as a sole phosphorus source. The cells were suspended in concentration of 5 mg. wet weight, per ml. Phosphorus-deficient medium is a modified Sauton medium, in which KCl was substituted for K_2HPO_4. The cell suspensions were then incubated at 37°C. At appropriate intervals, an aliquot of the culture was taken out and cells were washed in saline five times until washing solution showed no significant radioactivity. The cells thus obtained were then resuspended in $M/15$ non-labeled phosphate buffer (pH 7.1) to obtain the cell suspensions of 40 mg. wet weight, of cells per ml. and incubated at 37°C. During this incubation period the course of change in the radioactivity of various cellular fractions were followed. Thus, at appropriate time intervals, an aliquot (4.0 ml.) of cell suspension was washed in distilled water four times and the cells were fractionated according to the procedure of Schneider (*1*). Two-tenth mililiters sample of each fraction was placed in a stainless steel planchet and heated to dryness for the estimation of radioactivity using Geiger-Mueller counter. Radioactivity was expressed as count per minute (c.p.m.) per mg. dry weight, of cells.

Amounts of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were determined in nucleic acid fraction by diphenylamine reaction (*3*) and orcinol reaction (*4*), respectively. Amount of total protein was determined in extracts (extracted two times) by incubating bacterial cells in N KOH at 37°C for 24 hours, followed by determination using Folin-Ciocalteu's phenol reagent (*5*).

RESULTS AND DISCUSSION

Radioactive phosphorus incorporated into cells was released from cells by incubation of the radioactive cells in nonradioactive phosphate solution. Radioactivity of nucleic acid fraction was found to decrease, while that of acid-soluble fraction increased (Table I). Radioactivity remained almost unchanged in phosphatide fraction. Response of protein varied with the duration of time of P^{32}-incorporation. A considerable fraction of this fraction obtained from those cells, which were pretreated with P^{32} for less than 3 hours, was released by the incubation in nonradioactive phosphate medium. In contrast, that of the protein fraction of those cells, which had been made in contact with P^{32} for more than 5 hours, remained unchanged (Table I).

During the 5-hour incubation period in P^{32}-containing medium, the amounts of DNA, RNA and total protein in cells remained unchanged so far determined by the methods described above (Table II). No growth was observed throughout this period so far determined by turbidity.

In cells that incorporated P^{32} for the period of less than 3 hours, stability of protein fraction seemed to be parallel with that of nucleic acid fraction. As deduced from Table I, it

TABLE I

Stability of Radioactivity of Various Cellular Fractions Obtained from Cells Which Were Previously Subjected to Incorporte P^{32}-Orthophosphate for Various Time Intervals

Duration of Labeling[1]	Cellular Fraction[2]	Radioactivity as c.p.m. per mg. (dry weight) of cells			
		Time of incubation in P^{32}-free phosphate buffer			
		0 hr	1 hr	3 hrs	5 hrs
1 hr	Acid-Soluble	2,415	2,320	3,315	3,750
	Phosphatide	176	156	165	172
	Nucleic Acid	2,085	1,865	836	825
	Protein	154	114	74	104
3 hrs	Acid-Soluble	3,480	3,210	3,925	4,305
	Phosphatide	213	209	213	206
	Nucleic Acid	2,300	2,360	948	910
	Protein	155	134	76	76
5 hrs	Acid-Soluble	3,250	3,710	4,200	4,430
	Phosphatide	376	369	426	386
	Nucleic Acid	2,500	2,290	1,520	1,400
	Protein	311	357	331	329
24 hrs	Acid-Soluble	4,520	4,640	4,650	4,900
	Phosphatide	640	640	610	654
	Nucleic Acid	4,900	4,750	4,620	4,500
	Protein	678	653	670	662

1) Time in hours during which cells (5 mg./ml.) was incubated in media containing P^{32}-orthophosphate (1.5 uc/ml.).

2) Fractionated according to the procedure of Schneider (*1*).

TABLE II

Amounts of DNA, RNA and Total Protein in Cell during Various Periods of P^{32}-Incorporation

	Time of incubation in P^{32}-phoshate-containing medium			
	0 hr	1 hr	3 hrs	5 hrs
	μg. per mg. dry wieght, of cells			
DNA	19.0	18.5	20.0	17.5
	μg. per mg., dry weight, of cells			
RNA	119.5	124.0	130.4	119.4
	μg. per mg., dry weight, of cells			
Protein	172	170	173	174

was also found that, as the duration of incorporation became longer than 3 hours, stabilization of protein fraction occurrred earlier than that of nucleic acid fraction.

It may be probable to consider that this labile compound might be a residual fraction of RNA or polyphosphate which was left unextracted in protein fraction on extracting cells with hot TCA. If this might be the case the protein fraction obtained from the cells preincubated in P^{32}-containing medium for 5 hours also should contain such a labile fraction, since the nucleic acid fraction still remained unstable at this incubation period. However, this was found not to be the case as shown in Table I. The above possibitilty thus appears to be excluded.

SUMMARY

In the early phase of phosphorus incorporation, a labile phosphorus compound showing active phosphorus turnover appeared in protein fraction obtained by the Schneider's procedure. In later phase, however, this labile compound was shown to be absent.

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Bacterial Formation of Glutamic Acid from Acetic Acid

II. Formation of Glutamic Acid from C^{14}-Labelled Acetic Acid in Brevibacterium flavum, No. 2247

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In the preceding paper of this series (*1*), it was shown that α-ketoglutarate or glutamate, the latter in the presence of ammonium ions, was formed aerobically from acetate by the resting cells of *Brevibacterium flauum*, No. 2247, and that the cell-free extracts were capable of catalysing the formation of acetyl-CoA from acetate (*2*, *3*) and the subsequent condensation of acetyl-CoA either with oxaloacetate to form citrate (*4*) or with glyoxylate to form malate (*5*). It was also described previously that isocitratase (*6*, *7*) and almost all enzymes of the TCA cycle were present (*1*, *8*). Therefore, a modified TCA cycle with an additional "glyoxylate by-pass" (*9*) has been suggested as a possible pathway of glutamate formation from acetate in this bacterium. The postulated pathway can also explain the incorporation of C^{14} of $C^{14}O_2$ into α-carboxyl carbon of glutamate formed from acetate by the resting cells, provided that the isotope-exchange reactions between C_4 compounds and CO_2 are present. In fact, evidence has been obtained for the presence of the oxaloactic carboxylase of Utter and Kurahashi (*10*) and the malic enzyme of Ochoa (*11*) in this organism (*12*).

The present paper deals with the pathway of glutamate formation from C^{14}-labelled acetate in *B. flavum*.

Because of the relative abundance in the organisms, of the ease of isolation, and of the *in vivo* interconversion with α-ketoglutarate, which is a key compound in the TCA cycle, glutamate has been considered to be a specifically useful compound to study the general metabolic pathway in the organisms by the isotopic tracer techniques. Many studies along this line have been described with microorganism (*13–18*), rats (*19–24*), and cow (*25*). Under certain conditions, particularly when several pathways are involved, however, the isotopic data obtained from glutamate have been difficult to interpret.

The results reported in the present paper can well be explained on the assumption that the above mentioned cycle is the major pathway of the incorporation of acetate carbon into glutamate in the intact cells of *B. flavum*.

METHODS

Isotopic Materials—Sodium acetate-1- and -2-C^{14} were obtainee from the Daiichi Pure Chemicals Co., Ltd., and L-glutamic acid-U-C^{14} from the Institute of Applied Microbiology, University of Tokyo, Tokyo. The latter was further purified by the method described below.

Washed Cell Suspension—The cells grown in the medium described previously (*8*) containing glucose and urea as the carbon and nitrogen source were harvested after 24 hours centrifugation, washed with 0.2% KCl, suspended in 0.05 *M* phosphate buffer, pH 7.5, aerated at 30°C for 2 hours, and resuspended in 0.01 *M* phosphate buffer, pH 7.5.

Formation of C^{14}-Glutamate from C^{14}-Acetate—The experiments on the formation of C^{14}-glutamate from acetate-1- and -2-C^{14} were performed in conventional Warburg vessels under air at 37°C. The reaction mixture consisted of 150 μmoles of sodium and potassium phosphate, pH 7.5, 70 μmoles of NH_4Cl, about 100 μmoles of potassium acetate (giving 2.32 and 1.94×10^6 c.p.m. for acetate-1-C^{14} and -2-C^{14}, respectively), 1 ml. (1.28 mg. N) of washed cell suspension and water in a total volume of 5.0 ml. The center well of the vessel contained 0.2 ml. of 20% KOH and a piece of filter paper. At the end of the incubation period the reaction was stopped by tipping 1 ml. of 0.5 *N* HCl from the side arm. The reaction mixture

was allowed to stand for a while to ensure the complete absorption of CO_2 into KOH and then centrifuged. Glutamic acid formed was separated by two-dimensional paperchromatography (solvent: *n*-butanol-acetic acid-water (4 : 1 : 1), and phenol-water (10 : 2)) and its total radioactivity and specific radioactivity were measured as described in the previous paper (1). α-Ketoglutaric acid was extracted after conversion to 2,4-dinitrophenylhydrazone, isolated on a paperchromatogram (solvent: *n*-butanol-ethanol-0.5 *N* NH_4OH (7 : 1 : 2)), and its radioactivity was measured as described previously (*12*, *26*). The radioactivity incorporated into cells was also measured after the cells were centrifuged, washed once with cold water, and plated directly. Carbon dioxide absorbed into KOH was precipitated in the form of $BaCO_3$ and its radioactivity was measured as described previously (*26*).

Degradation of Glutamic Acid—The isotope in carbon-1 was determined in the form of $BaCO_3$ after the treatment of the glutamate with glutamic decarboxylase of *E. coli* "Crookes" dried cells (*1*). The relative specific activity of carbon-5 and of the whole molecule could be determined after trapping $C^{14}O_2$ arising from the Schmidt degradation of glutamate (*21*, *27*).

RESULTS AND DISCUSSION

Metabolism of C^{14}-Labelled Acetate—Experiments were performed in Warburg vessels to determine the distribution of C^{14} during the metabolism of radioactive acetate, and to prepare C^{14}-glutamate for determination of the intramolecular distribution of C^{14}. After 2 hours aeration, the resting cells were aerobically incubated with approximately 100 μmoles of acetate-1- or -2-C^{14} in two conventional Warburg vessels for 5 hours. The cells, carbon dioxide, glutamic acid as well as some other non-volatile products in the medium were analyzed for radioactivity as shown in Table I. The total recovery of C^{14} was about 90 per cent. Since the oxygen absorption was still observed at the end of reaction period, this deficit would be attributed to the incomplete consumption of the volatile substrate. The occurrence of oxidative assimilation of acetate was also shown by a considerable amount of radioactivity incorporated into the cells, which could not be due to the contamination of outside medium from the quantitative view, and would explain the deficit of the carbon balance observed in the oxidation of acetate previously reported.

Table I

Metabolism of C^{14}-Labelled Acetate by Resting Cells[1]

Product	Radioactivity derived from Acetate-1-C^{14}	Acetate-2-C^{14}
	$\times 10^5$ c.p.m.	
Acetate added	23.2	19.4
Glutamate	3.07	5.84
α-Ketoglutarate	0.09	0.17
Cells	0.76	1.51
Carbon dioxide	15.9	11.0
Unknown spots[2]	0.11	0.21
Recovery	86%	96%
	Specific radioactivity	
	$\times 10^4$ c.p.m./μmole	
Acetate added	2.17±0.02	1.86±0.07
Glutamate formed	3.54±0.07	5.35±0.11

1) Reaction mixture contained 100 μmoles of potassium acetate, 150 μmoles of phosphate buffer, pH 7.5, 70 μmoles of NH_4Cl, 1.28 mg.N of washed cell suspension, and water in a total volume of 5.0 ml. Shaken under air at 37°C for 5 hours.

2) Unknown spots shown in the radioautogram of two-dimensional paperchromatogram of non-volatile products.

Distribution of Radioactivity in Glutamate from Specifically Labelled Acetate—For the determination of the C^{14} distribution, glutamate thus formed was degraded by the methods described above after paperchromatographic purification (Table II, III).

Since enzymic decarboxylation of α-carboxyl group of L-glutamic acid proceeds quantitatively, the total radioactivity of CO_2 formed by the action of decarboxylase was measured. The yield of CO_2 from carbon-5 released by the Schmidt reaction on glutamic acid was still lower than the theoretical value (50~90%), even if 3-fold excess of azide was used, and hence the specific activity of the CO_2 absorbed in CO_2-free alkaline solution was measured. That the results obtained with uniformly labelled L-glutamic acid are in good agreement with those expected, indicates

TABLE II

Determination of Labelling in Carbon-5 of Glutamic Acid Derived from C^{14}-Labelled Acetate[1]

Label in acetate	Glutamic acid	$BaCO_3$ from carbon-5			Label in C-5
	Specific activity	Weight	Radioactivity[3]	Specific activity	
	$\times 10^4$c.p.m./mmole	mg.	c.p.m.	$\times 10^4$c.p.m./mmole	%
1-C^{14}	3.26±0.08	58.17	5,544	1.88	
	3.26±0.08	68.34	6,433	1.86	
			mean	1.87±0.01	57.4±1.7
2-C^{14}	3.59±0.02	68.10	254.4	0.074	
	3.59±0.02	93.52	375.0	0.079	
			mean	0.076±0.003	2.13±0.09
(U-C^{14})[2]	6.82±0.11	34.77	2,396	1.36	
	6.82±0.11	48.92	3,210	1.30	
			mean	1.33±0.03	19.5±0.8

1) Glutamic acid was degraded by Schmidt reaction (*21*, *27*).
2) Uniformly labelled glutamic acid was used.
3) Correction for self-absorption was made.

TABLE III

Determination of Labelling in Carbon-1 of Glutamic Acid Derived from C^{14}-Labelled Acetate[1]

Label in acetate	Radioactivity		Label in C-1	Label in C-2, 3, 4[4]
	Glutamate added	$BaCO_3$ formed[3]		
	$\times 10^4$ c.p.m.	c.p.m.	%	%
1-C^{14}	1.64±0.04	6,338		
	1.64±0.04	7,086		
	mean	6,712±374	41.0±3.3	1.6±5.0
2-C^{14}	1.81±0.01	2,686		
	1.81±0.01	2,574		
	mean	2,630±56	14.6±0.4	83.3±0.5
(U-C^{14})[2]	3.43±0.05	6,909		
	3.43±0.05	7,038		
	mean	6,974±65	20.3±0.4	60.2±1.3

1) Glutamic acid was decarboxylated by glutamic decarboxylase of *E. coli* "Crookes" (*1*).
2) See Table II.
3) Correction for self-absorption was made.
4) Determined by substracting the value of carbon-1 and -5 from 100%.

the validity of the methods used in these experiments.

As shown in Table IV, carbon-2, 3, 4 and carbon-5 of the glutamic acid are derived almost exclusively from carbon-2 and carbon-1 of the substrate, acetic acid, respectively, whereas both two carbons of the acetate molecule participate in the formation of carbon-1 of the glutamate.

Tables II, III, and IV show that in *B. flavum* acetate-1-C^{14} labels only carbon-1 and

TABLE IV

Relative Specific Activity of Carbon in the Acetate Added and the Glutamate Formed

	Relative specific activity[1]			Carbon source Acetate C-1, C-2
	A	B	A+B	
Acetate added				
Carbon-1	100	0	100	
,, -2	0	100	100	
Glutamate formed				
Carbon-1	66.8±7.2	41.9±3.6	109±11	62 : 38
,, -2	0.9±2.7[2]	79.7±5.2	81±8	1 : 99
,, -3				
,, -4				
,, -5	93.6±5.4	6.0±0.6	100±6	94 : 6

1) Relative specific activity of carbon-x : (% in carbon-x) (specific activity of glutamate)/(specific activity of acetate added). (x : 1, 2, 3, 4 or 5)
2) Mean value of the three carbon atoms, C-2, 3, 4.

-5 of glutamic acid. This labelling pattern of glutamic acid agrees with that expected from the operation of the TCA cycle. However, since the TCA cycle is a means whereby acetate is oxidized to carbon dioxide, mechanisms must operate whereby the acceptor for C_2 units can be synthesized from acetate when glutamate is produced from acetate as a sole carbon source. It is feasible, for instance, that C_4 units could be synthesized from CO_2 by mechanisms such as are operative in photosynthetic (*28*) or autotorophic organisms (*29*). This possibility is rendered unlikely, however, by the present results and by the previously reported finding that glutamate formed is labelled almost exclusively in the carbon-1 when $C^{14}O_2$ is added to the reaction system (*1*).

There are still other possibilities. Namely, evidence has been found for the existence of (a) the condensation of acetate with CO_2 or derivatives thereof in *Clostridium kluyveri*, *E. coli*, and in *Rhodospirillum rubrum*; (b) the exchange of the carbon-1 of pyruvate with CO_2 in *C. butyricum*, *C. acidiurici*, *A. aerogenes*, *E. coli*, *Micrococcus pyogenes*, and in a rumen organism LC; (c) the incorporation of a C_1 unit into pyruvate in *E. coli*, and *Micrococcus lactilyticus* (*30*). Therefore, it could thus be envisaged that C_4 compounds are synthesized by the successive carboxylation of acetate to give pyruvate and, in turn, of pyruvate to give malate. The latter reaction has beed demonstrated in the extracts of *B. flavum* (8, 12). However, the present data are not consistent with this possibility.

The labelling patterns of glutamic acid derived from CH_3-$C^{14}OOH$ labelled "100" in the carboxyl group would become

HO_2C	$CHNH_2$	CH_2	CH_2	CO_2H
50	0	0	0	100

by the sole operation of the TCA cycle. In fact such patterns were observed in yeast (*15*), *Penicillium chrysogenum* (*16*), rats (*21*), and cow (*25*).

The observed labelling patterns, however, show a higher radioactivity in the α-carboxyl group of glutamic acid than that predicted for the sole operation of the TCA cycle. This suggests the existence of some other pathway leading to an enhanced incorporation of the carboxyl group of acetate into the α-carboxyl group of glutamic acid. The operation of the "glyoxylate cycle", demonstrated in bacterial extracts, would lead to this effect. In this cycle, the labelled carbon from the carboxyl group of CH_3-$C^{14}OOH$ enters the metabolic routes not only by condensation with oxaloacetate to form citrate, but also by condensation with glyoxylate (derived from isocitrate) to form malate. Recycling of the labelled carbon through these reactions would eventually lead to glutamic acid labelled in such a way as:

HO_2C	$CHNH_2$	CH_2	CH_2	CO_2H
100	0	0	0	100

The amount of radioactivity in the α-carboxyl group of glutamic acid, which was higher than that expected from the sole operation of the TCA cycle, but lower than that expected from the sole operation of the "glyoxylate cycle", agrees well with the view that "glyoxylate cycle" operates to compensate intermediates drained from the TCA cycle during the biosynthesis of glutamic acid. The C^{14} distribution in the glutamic acid acid formed can be accounted for, under the following conditions: (a) The "metabolic ratio" of isocitrate, to α-ketoglutarate *versus* to glyoxylate plus succinate, k: $(1-k)$, is kept constant throughout the experiment period. So is the "metabolic ratio" of α-ketoglutarate to succinate *versus* to glutamate, l: $(1-l)$. (b) The malate maintains the symmetrical distribution of C^{14} through the complete equilibration with fumarate. (c) The glutamate formed is readily excreted from the cell and is not exchangeable with the intracellular α-ketoglutarate. (d) The total amount of intermediates remains constant and is much smaller than that of glutamate formed. (e) No extraneous carbon enters the cycles. Under these conditions the C^{14} distribution in any given intermediate of the cycle depends on the number of "turns" the cycle has made and the glutamic acid arising from amination of α-ketoglutaric acid would theoretically have the C^{14} distribution shown in Table V.

TABLE V

Theoretical C^{14} Distribution in Glutamic Acid Arising from the "TCA-Glyoxylate Cycle" during Metabolism of Acetate Containing 1 Unit C^{14} on the Carboxyl Carbon

No. of "turn"	Radioactivity in glutamic acid carbon formed after each additional one turn			Amount of glutamic acid formed after each one after
	C-1	C-2,3,4	C-5	
1	0 ($\equiv A_1$)	0	1	$k(1-l)I$
2	1/2 ($\equiv A_2$)	0	1	$k(1-l)IF$
3	$1/2+(1-k)A_2/F$ ($\equiv A_3$)	0	1	$k(1-l)IF^2$
.	.	.	.	.
.	.	.	.	.
n	$1/2+(1-k)A_{n-1}/F$ ($\equiv A_n$)	0	1	$k(1-l)IF^{n-1}$

F: $kl+2(1-k)$

I: Total amount of intermediates in the cells at initial state.

k, l: See text.

From the assumption (d), the following relation can be obtained:

$$kl+2\ (1-k)=1$$

Thus, the number average of radioactivity observed in carbon-1 of glutamate arising from the "TCA-glyoxylate cycle" during metabolism of acetate containing 1 unit C^{14} on the carboxyl carbon would be;

$$\bar{A}_{(1)}=1/(2k),$$

and that of glutamate formed from methyl-labelled acetate would be;

$$\bar{A}_{(2)}=1-\bar{A}_{(1)}=1-1/(2k)$$

From the experimental values for $A_{(1)}$ and $A_{(2)}$. these "metabolic ratios" would be determined as follows:

$$k\text{: } (1-k)=4:1$$
$$l\text{: } (1-l)=3:1$$

In other words, the C^{14} distribution observed in glutamate would be explained by the "TCA-glyoxylate cycle" when these "metabolic ratios" have above values.

The only difference between the pathway postulated previously for glutamate formation (Fig. 1) and the above mentiond "TCA-glyoxylate cycle" for acetate metabolism is the presence of the reaction to form succinate from α-ketoglutarate. It should be noted that α-ketoglutarate was produced from acetate as

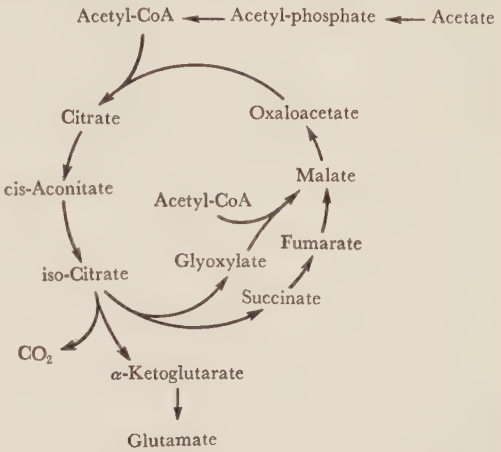

FIG. 1. Postulated pathway of glutamate formation from acetate (*1*).

an end product and was not metabolized aerobically nor anaerobically by intact cells of this organism (*1*, *31*). Since the above mentioned "TCA-glyoxylate cycle" cannot explain these results, it cannot be accepted as a possible pathway of glutamate formation in this case.

However, another explanation would be possible for the isotopic data. The main pathway for glutamate formation from acetate in *Brevibacterium flavum* has been suggested in the previous paper to be a modified TCA cycle with an additional "glyoxylate by-pass" (Fig. 1), in which one molecule of glutamic acid and of carbon dioxide are derived from every three molecules of acetic acid. However, since higher yield of carbon dioxide is always observed in the metabolism of acetate in this organism, some other additional pathway or pathways must be operative by which carbon dioxide may be produced from acetate or its metabolic intermediates. On the other hand, in the case of glutamate formation from acetate-2-C^{14}, C^{14} should not appear at the carbon-1 position as a result of the sole operation of this cycle but the appering of C^{14} in the carbon-1 could be accounted for by the sequence shown in Fig. 2. The acetate formed along this devious route would contain C^{14} on both

carbon atoms and, upon re-entering the cycle by condensation with glyoxylate, would lead to the formation of glutamic acid with C^{14} at

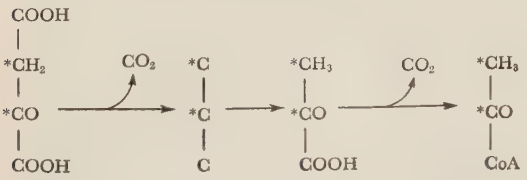

Fig. 2. Acetyl-CoA formation by degradation of oxaloacetate.

The C^{14} distribution of oxaloacetate is shown for that formed during metabolism of acetate-2-C^{14}.

the C-1 position It might be expected that the same radioactivity as noted in the carbon-1 of glutamate, synthesized in the presence of reformed carboxyl-labelled acetate, is detected also in the carbon-5. That this is not observed actually may be due to the synthesis of acetyl-CoA by two routes: (a) From acetic acid by the action of acetokinase and phosphotransacetylase (*1*), and (b) from pyruvic acid by the action of pyruvic dehydrogenase (*32*). It seems likely that these reactions occur at different sites in the cell, and carbonyl-labelled acetyl-CoA derived from pyruvate is preferentially utilized by malate synthetase. The presence of two such pools of differently labelled acetyl-CoA would then prevent the completely equall labelling in both two carboxyl groups of the glutamate derived therefrom.

Thus, the C^{14} distribution in glutamic acid formed can be accounted for by these hypotheses as well as by the operation of the "TCA-glyoxylate" cycle. The theoretical C^{14} distributions in the glutamic acid drained from the cycle after each additional one turn are shown in Table VI, where p: $(1-p)$ is "metabollic ratio" of isocitrate, to glyoxylate plus succinate *versus* to α-ketoglutarate, and q: $(1-q)$ is that of oxaloacetate, to citrate *versus* to C_3 unit. From the assumption (d), the following relation can be obtained:

$$2pq=1$$

Thus, the number average of radioactivity observed in carbon-1 of glutamate arising from the previously postulated cycle plus oxaloacetate degradation route during metabolism of acetate containing 1 unit C^{14} on the carboxyl carbon would be

$$\bar{A}_{(1)}=q_2,$$

and that of glutamate formed from methyl labelled acetate would be

$$\bar{A}_{(2)}=1-\bar{A}_{(1)}=1-q.$$

Therefore, from the data for $\bar{A}_{(1)}$ and $\bar{A}_{(2)}$, these "metabolic ratios" would be determined as follows:

$$p:(1-p)=4:1$$
$$q:(1-q)=3:2$$

In other words, the previously postulated cycle for the glutamate formation for acetate can account for the C^{14} distribution in the glutamic acid by adding a degradation route of oxaloacetate shown in Fig. 2.

It is of interest that the route shown in Fig. 2 has been postulated as a part of dicarboxylic acid cycle for the oxidation of glyoxylate by Kornberg and Sadler (*33*).

Table VI

Theoretical C^{14} Distribution in Glutamic Acid Arising from the Previously Postulated Pathway with an Additional Oxaloacetate Degradation Route during Metabolism of Acetate Containing 1 Unit C^{14} on the Carboxyl Carbon[1)]

No. of "turn"	Radioactivity in glutamic acid carbon formed after each additional one turn			Amount of glutamic acid formed after each one turn
	C-1	C-2,3,4	C-5	
1	0 ($\equiv A_1$)	0	1	$(1-p)$I
2	1/2 ($\equiv A_2$)	0	1	$(1-p)$IL
3	$1/2[A_2+1-(1-q)/L]$ ($\equiv A_3$)	0	1	$(1-p)IL^2$
.	.	.	.	.
.	.	.	.	.
n	$1/2[A_{n-1}+1-(1-q)/L]$ ($\equiv A_n$)	0	1	$(1-p)IL^{n-1}$

1) L: $2pq$
I: See Table V.
p, q: See text.

Summary

1. Labelled glutamic acid was produced from acetate-1-and -2-C^{14} in the presence of NH_4Cl and phosphate salt by washed cell sus-

pension of *Brevibacterium flavum*, No. 2247. The relative specific activities of glutamate to the substrate acetate were 1.63 and 2.88, respectively. The percentages of radioactivity in the carbon-1 were 41 and 14.6 per cent, and those in the carbon-5 were 57 and 2.1 per cent respectively.

2. From these results the main pathway of glutamate formation from acetate used as the sole carbon source in the intact cells was discussed and the isotopic data could be explained by the previously postulated pathway, under some conditions.

The authors are indebted to Prof. F. Egami and Dr. T. Yamada of the University of Tokyo, and Dr. H. Oeda and Mr. S. Motozaki of our laboratory for the helpful discussion and encouragement during the course of this work.

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Bacterial Formation of Glutamic Acid from Acetic Acid

III. Incorporation of Labelled Acetate and Kinetic Relationships of the Metabolites in Brevibacterium flavum, No. 2247

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In the previous papers (*1–5*), in which glutamic acid formation in resting cells and cell-free extracts of *Brevibacterium flavum* was dealt with, several remarkable features of this bacterium were described. First, enzymes of the acetlyCoA formation, the TCA cycle, and the "glyoxylate by-pass" (*6*) were found to be present in the cell-free extracts, and from these results, a modified tricarboxylic acid cycle with an additional "glyoxylate by-pass" has been suggested as the possible pathway of glutamate formation from acetate in this bacterium. Secondly, it was shown that the incorporation of C^{14} of $C^{14}O_2$ into α-carboxyl carbon of glutamate formed from acetate as well as the distribution of C^{14} in the glutamate formed from acetate-1-, and -2-C^{14} could be well explained by the postulated pathway.

Although the above mentioned results might well indicate the possible operation of the "glyoxylate by-pass" in the glutamate formation reactions of the intact cells, more direct evidence would be required for recognizing the physiological significance of such a mechanism in the intact cells.

Some kinetic approaches have been made in order to elucidate the nature of the reactions occuring in *Penicillium chrysogenum*, *Pseudomonas fluoresces*, a *Corynebacterium* sp., and *E. coli* grown on acetate, in *Pseudomonas oxalaticus* OXI grown on formate or oxalate, and in *E. coli* M22–64 grown on glycollate (*7–13*).

This paper constitutes a report of an extension of the experimental kinetic analysis of this system, and the results obtained with intact organisms are in accordance with those as demonstrated in cell-free extracts that the "glyoxylate by-pass" of the tricarboxylic acid cycle is operative (*1*).

METHODS

Washed Cells—Brevibacterium flavum No. 2247 was grown aerobically at 30°C for 24 hours in a medium described previously containing glucose and urea as a carbon and nitrogen source, respectively. The cells were harvested by centrifugation and washed twice with 0.2% KCl.

Isotopic Material—Sodium acetate-2-C^{14} was obtained from the Daiichi Pure Chemicals Co., Ltd. The radiochemical purity was confirmed by paperchromatography in ethanol-water-28% NH_4OH (80 : 60 : 4).

Incorporation of Acetate-2-C^{14}—Washed cells were suspended in a medium containing 0.01 *M* potassium acetate and 0.02 *M* phosphate buffer, pH 7.5. The suspension (0.024 mg. N/ml.) was shaken at 30°C for one hour. One ml. of the acetate-2-C^{14} solution, containing 46 μmoles of acetate with 200 μc. of C^{14}, was added at zero time to 10 ml. of the above suspension. After rapid manual swirling, samples (approximately 1 ml.) were pipetted out at known times into 3 ml. of hot absolute ethanol (80°C). The hot ethanolic (75%) suspensions were left at 80°C for 15 minutes, cooled and cetrifuged at $10{,}000 \times g$. The clear supernatant solutions were evaporated to dryness at 60°C. The residues were dissolved in 0.2 ml. of 20% (v/v) aqueous ethanol and analysed by descending two-dimensional paperchromatography (*1*). The radioactive compounds were located by radioautography, and the C^{14} content was determined with a mica end window β-counter tube after being eluted with water.

RESULTS AND DISCUSSION

When the cells were incubated with acetate-2-C^{14} for periods of time ranging

from 3 seconds to 150 seconds, two-dimensional chromatography and radioautography of the ethanol-soluble fractions obtained from each sample of the suspension showed that C^{14} from acetate rapidly appeared in many of the intermediates of the TCA cycle and in amino acids, such as aspartate and glutamate, directly derived therefrom. An example of the radioautograms was reproduced as shown in Fig. 1. Since the separation of malate from an unknown compound A, and

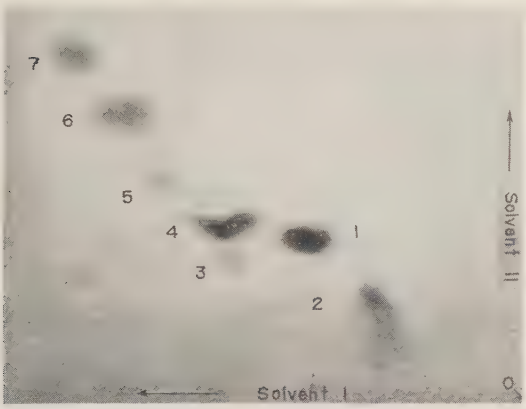


Fig. 1. Radioautogram of two-dimensional paperchromatogram of ethanol-soluble fraction obtained from *Brevibacterium flavum* 2247 incubated with acetate-2-C^{14}.

After one hour of preincubation with 0.01 *M* potassium acetate, acetate-2-C^{14} (46 μmoles, 200 μc) was added at zero time to 10 ml. of bacterial suspension (0.024 mg. N/ml.). One ml. of the reaction mixture was pipetted into 3 ml. of hot ethanol after one minute of aerobic incubation.

Solvent I: phenol-formic acid-water, solvent II: *n*-butanol-propionic acid-water (*1*).

Spot 1: citric acid; 2: aspartic acid; 3: glutamic acid and α-ketoglutaric acid; 4: malic acid and unknown compound A; 5, 6: unknown compounds B, C; 7: succinic acid; O: "origin",

of glutamate from α-ketoglutarate was incomplete on the first two-dimensional paperchromatogram, these mixtures were further rechromatographed in *n*-butanol-formic acid-water (4:1.5:1) and in *n*-butanol-acetic acid-water (4:1:1), respectively. Radioactive compounds detected in this manner were identified by their relative R_f values on the two-dimensional chromatograms, and then by co-chromatography with known compounds. The solvents used were *n*-butanol-acetic acid-water (4:1:1) and phenol-water (10:2) for amino acids, *n*-butanol-formic acid-water (4:1.5:1) for organic acids, and *n*-butanol-ethanol-0.5 *N* NH_4OH (7:1:2) for 2,4-dinitrophenyl-hydrazones of keto acids (*1*, *4*). Some unidentified compounds (A, B, C) were observed which might be the intermediates in fatty acid and steroid synthesis.

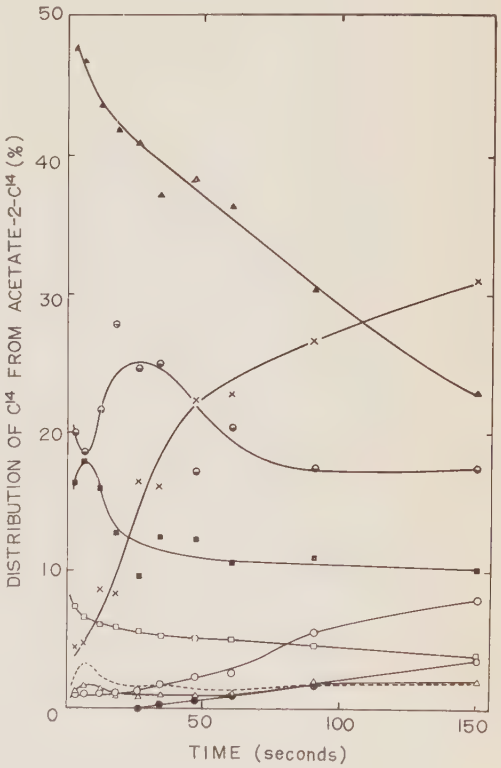


Fig. 2. Percentage distribution of C^{14} incorporated from labelled acetate among the constituents of the ethanol soluble fraction of *B. flavum*.

Conditions were similar to those described in Fig. 1. —×—; citrate; —●—, glutamate; —○—, α-ketoglutarate; —□—, malate; —■—, succinate; —△—, aspartate; —▲—, unknown compound A; —◒—, unknown compound B; · · ·, unknown compound C.

The percentages of C^{14} contributed by each constituent of the sample were plotted against time and the curves obtained were shown in Fig. 2. From the nature of these

isotopic distribution curves and of the various compounds concerned, it would be possible to determine their place in the complex synthetic system which must exist in the cell. The technique of our kinetic experiment can be applied to a steady state system in which the mass concentration in each of the compounds remains constant. Let us consider a simple reaction chain that consists of a sequence of first order reactions. The system to be discussed may be represented as

$$P_1 \underset{k_1'}{\overset{k_1}{\rightleftharpoons}} P_2 \underset{k_2'}{\overset{k_2}{\rightleftharpoons}} P_3 \underset{k_3'}{\overset{k_3}{\rightleftharpoons}} \cdots \underset{k_n'}{\overset{k_n}{\rightleftharpoons}} \cdots$$

(k, k': rate constant).

Assuming an initial concetration p_0 of the radioctive P_1, which, after time t, has reacted to give a concentration p_2 of that of P_2, p_3 of that of P_3, $\cdots$, p_n of that of P_n, $\cdots$, leaving a concentration p_1 of that of P_1, the rate equations may be formulated as

$$\begin{aligned}
dp_1/dt &= -k_1p_1 + k_1'p_2 \\
dp_2/dt &= k_1p_1 - (k_1' + k_2)\ p_2 + k_2'p_3 \\
dp_3/dt &= k_2p_2 - (k_2' + k_3)\ p_3 + k_3'p_4 \\
&\vdots \\
dp_n/dt &= k_{n-1}p_{n-1} - (k'_{n-1} + k_n)\ p_n + k_n'p_{n+1} \\
&\vdots
\end{aligned}$$

$$(t=0;\ p_1=p_0,\ p_2=p_3=\cdots=p_n=\cdots=0)$$

Although the integration of these equations may be carried out easily, the resulting expression is quite complex. On the other hand, since P_n, a function of time t, can be developed into the "Taylor's series"

$$p_n(t) = p_n(0) + p_n'(0)\ t/1! + p_n''(0)\ t^2/2! + \cdots + p_n^{(i)}(0)\ t^i/i! + \cdots,$$

where, under the above described conditions,

$$\begin{aligned}
&p_n(0) = p_n'(0) = p_n''(0) = \cdots = p_n^{(n-2)}(0) = 0, \\
&p_n^{(n-1)}(0) = k_{n-1}\, p_{n-1}^{(n-2)}(0) \\
&p_{n-1}^{(n-2)}(0) = k_{n-2}\, p_{n-2}^{(n-3)}(0) \\
&\vdots \\
&p_2'(0) = k_1p_0,
\end{aligned}$$

the following simplified expression may be valuable at the initial stages of reaction;

$$p_n(t) = A_{n-1}t^{n-1} + 0(t^n),\ (n=1, 2, 3, \cdots),$$

where

$$A_{n-1} = k_1k_2k_3 \cdots k_{n-1}p_0/(n-1)!\ (>0),$$

and $0(t)$ is a function consisting of the n th and higher power of t.

Then, the fraction F_r of the radioactivity of P_r among an arbitrarily selected group of compounds (P_m, P_n, $\cdots$, P_z; $m < n \cdots < z$) is

$$F_r = p_r/(p_m + p_n + \cdots + p_z),$$

and the differential of F_r is

$$dF_r/dt = (dp_r/dt)/(p_m + p_n + \cdots + p_z) - [p_r \cdot d\ (p_m + p_n + \cdots + p_z)/dt]/(p_m + p_n + \cdots + p_z)^2$$

If $r \neq m$,

$$dF_r/dt = (r-m)\ (A_{r-1}/A_{m-1})\ t^{r-m-1} + 0\ (t^{r-m}),$$

and if $r=m$,

$$dF_r/dt = (m-n)\ (A_{n-1}/A_{m-1})\ t^{n-m-1} + 0\ (t^{n-m}).$$

Therefore, at the earliest stages of reaction,

if $r \neq m$, $dF_r/dt > 0$

and if $r=m$, $dF_1/dt < 0$.

In other words, if the percentage distribution of C^{14} among an arbitarily selected group of compounds is plotted, that one which precedes the others in the reaction sequence will be the one having a negative initial slope whereas all the others will have positive slopes.

A similar consequence as above would be obtained even in the more complex reaction chain having some branched points as follows:

$$\begin{array}{l}
P_1 \rightleftharpoons P_2 \rightleftharpoons P_3 \rightleftharpoons \cdots \\
\quad \downarrow\uparrow \\
\quad\ Q_3 \rightleftharpoons Q_4 \rightleftharpoons \cdots \\
\qquad\quad \rightleftharpoons P_n \rightleftharpoons P_{n+1} \rightleftharpoons P_{n+2} \rightleftharpoons \cdots \\
\qquad\qquad\quad \downarrow\uparrow \\
\qquad\qquad\quad R_{n+1} \rightleftharpoons R_{n+2} \rightleftharpoons \cdots
\end{array}$$

Thus, the one having a nagative initial slope will be that one (Q_i) which occupies the nearest site in the reaction chain against the starting material (P_1), among an arbitrarily selected group of componds (O_l, P_j, P_k, R_l, Q_m, $\cdots$; $i < j < k < l < m < \cdots$), without regard for the relationship between precursor and product in a single reaction sequence (for example, Q_i is not a precursor of P_j, P_k, R_l, $\cdots$, while it is that of Q_m).

It is of interest to examine the relationship between the compounds identified above.

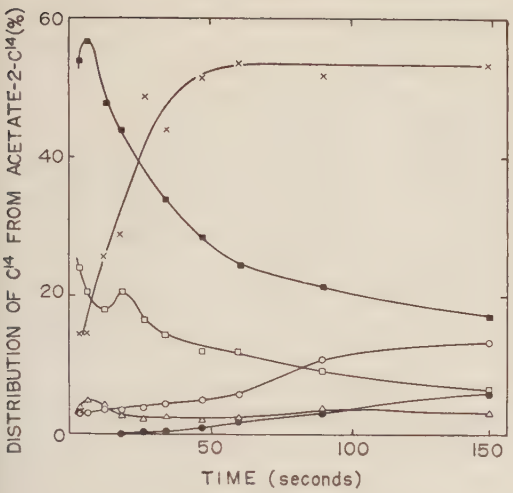


FIG. 3. Percentage distribution of C^{14} incorporated from labelled acetate among the intermediates of the TCA cycle, glutamate, and aspartate.

Conditions were similar to those described in Fig. 1. —×—, citrate; —●—, glutamate; —○—, α-ketoglutarate; —□—, malate; —■—, succinate; —△—, aspartate.

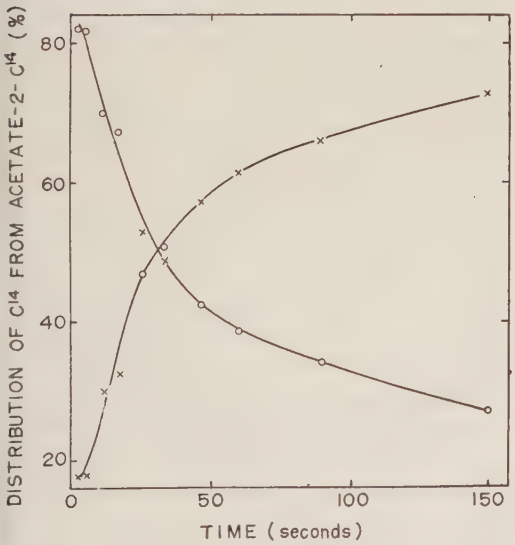


FIG. 4. Percentage distribution of C^{14} incorporated from labelled acetate among the C_6- plus C_5-compounds, and the C_4-compounds.

Conditions were similar to those described in Fig. 1. —×—, citrate + α-ketoglutarate + glutamate; —○—, malate + succinate + aspartate.

In order to determine the priority of their labelling, the percentage distribution of radioactivity among various sets of these compounds plotted against time, as shown in Fig. 3, 4, 5, 6, and 7. Fig. 3 indicates that

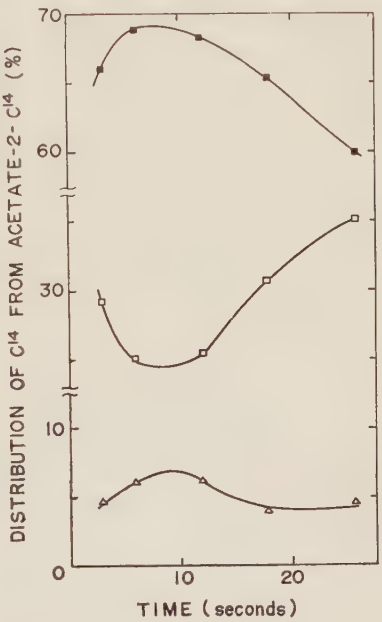


FIG. 5. Percentage distribution of C^{14} incorporated from labelled acetate among succinate, malate, and aspartate.

Conditions were similar to those described in Fig. 1. —■—, succinate; —□—, malate; —△—, aspartate.

at a very early stage of incubation over 50 per cent of acetate-C^{14} was incorporated into succinate and 20 per cent into malate. The latter proportion rapidly decreased whereas labelled citrate increased rapidly to a steady level at which its contribution was approximately 54 per cent in each sample. As shown at the earliest stages of the incubation, C^{14} from acetate entered malate without passing through the stage of citrate. This means that acetate entered the TCA cycle by a route not involving a condensation with oxaloacetate to form citrate. This conclusion was further confirmed when the total radioactivity in the C_6- plus C_5-compounds and that in the C_4-compounds were plotted against

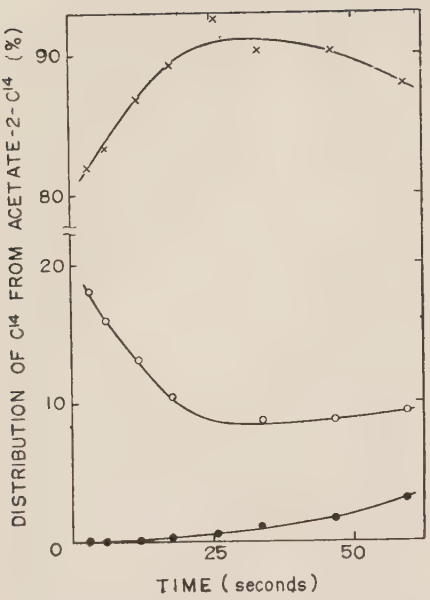


Fig. 6. Percentage destribution of C^{14} incorporated from labelled acetate among citrate, α-ketoglutarate, and glutamate.

Conditions were similar to those described in Fig. 1. —×—, citrate; —○—, α-ketoglutarate; —●—, glutamate.

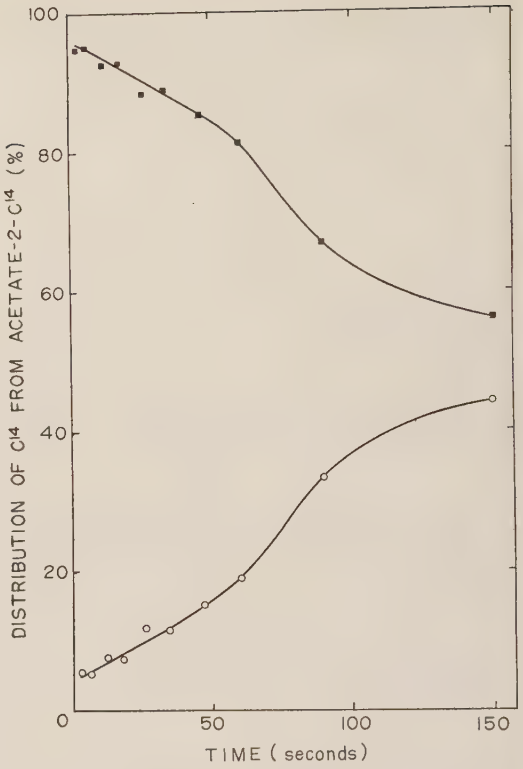


Fig. 7. Percentage distribution of C^{14} incorporated from labelled acetate between succinate and α-ketoglutarate.

Conditions were similar to those described in Fig. 1. —■—, succinate; —○—, α-ketoglutarate.

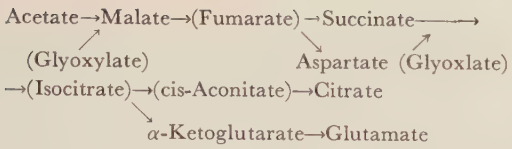
time (Fig. 4). Investigation with cell-free extracts of *B. flavum* provided additional support to this conclusion. The cell-free extracts were capable of catalysing the formation of acetyl CoA from acetate and the subsequent condesation of the acetylCoA either with oxaloacetate, yielding citrate, or with glyoxylate, yielding malate, which was derived from the aldol fission of isocitrate. The present results are in agreement with the operation of the "glyoxylate by-pass" *in vivo*, which has been demonstrated *in vitro* (*1*).

As shown in Fig. 5 and 6, malate and α-ketoglutarate appear to be labelled initially, while succinate, aspartate, citrate, and glutamate lag behind in each group of labelled compounds. Moreover, the negative slope of the C^{14} distribution curve of succinate in Fig. 7 indicates that the primary labelling product was succinate, and α-ketoglutarate became labelled later.

From these results it may be possible to construct a sequence of labelling of the compounds mentioned above as follows:

Acetate→Malate→Succinate→
↘
Aspartate
→α-ketoglutarate→Citrate
↘
Glutamate

It should be noted that α-ketoglutarate was produced from acetate as an end product and was not metabolized aerobically nor anaerobically by the intact cells of this organism (*1*, *2*). The C^{14} distribution in the glutamate formed from labelled acetate showed also that reductive carboxylation of succinate to form α-ketoglutarate may not occur in this organism (*2*). From these considerations, the labelling sequence may be expressed as:



This sequence of reaction is in agreement with the previously postulated pathway for the glutamate formation (*1*), with the exception that some reactions which are known to be reversible, are operative in reverse direction. The condesation reaction of acetyl-CoA with oxaloacetate to form citrate, which had been demonstrated in the cell-free extract, could not be detected in the present experiment. It would be due to the extremely small pool of oxaloacetate in the cell and to the incomplete aerobic condition that may be established in this experiment. The latter condition can also explain the change of direction of some reactions involved in the metabolic pathway.

SUMMARY

1. When the glucose grown, resting cells of *Brevibacterium flavum* were incubated with acetate-2-C^{14} for various periods of time, C^{14} from acetate rapidly appeared in citrate, α-ketoglutarate, succinate, malate, glutamate, and aspartate as revealed by the two-dimensional chromatography and radioautography of the ethanol-soluble fractions obtained from each sample of the suspension.

2. A method for kinetic analysis of the incorporation of labelled atom of a substrate into the metabolites was described in order to determine the metabolic pathway *in vivo*. When the percentage distribution of C^{14} among several particularly selected groups of the compounds was plotted against time, in order to determine the path of acetate metabolism, following reaction sequences were obtained, in due consideration of the biochemical characteristics of the compounds concerned: acetate, malate, succinate, α-ketoglutarate, glutamate; malate, aspartate; succinate, citrate.

3. The present results indicate the operation of the modified TCA cycle with an additional "glyoxylate by-pass" *in vivo*, which has been postulated from the results *in vitro* for the glutamate formation from acetate.

The authors are indebted to Prof. F. Egami and Prof. B. Maruo of the University of Tokyo, and Dr. H. Oeda and Mr. S. Motozaki of our laboratory for the helpful discussion and encouragement during the course of this work. The authors wish also to thank Dr. T. Yamada of the University of Tokyo for the helpful advices and discussion on the kinetic analysis.

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The Journal of Biochemistry, Vol. 49, No. 2, 1961

Metabolism of L-Lysine by Bacterial Enzymes

V. Glutaric Semialdehyde Dehydrogenase

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(Received for publication, October 12, 1960)

It was reported previously that an extract of a *Pseudomonas* bacteria adapted to lysine, metabolized DL-lysine to δ-aminovaleric acid. This in turn is transaminated (*1*). Subsequently the authors purified a δ-aminovaleric acid-glutamic acid transaminase from the extract, and identified one of the products of the reaction as glutaric semialdehyde (GSA*) (*2*).

The present paper describes the purification and the properties of this GSA dehydrogenase which converts GSA to glutaric acid with concomitant reduction of DPN.

METHODS AND MATERIALS

Preparation of the Acetone Dried Powder of Pseudomonas Bacteria Adapted to DL-*Lysine*—The strain of *Pseudomonas* used in this work was similar to that used previously (*1*). The methods used for culture of the bacteria and preparation of the acetone dried powder were also as reported previously (3).

Assay Method—The reaction mixture contained 60 μmoles of phosphate buffer (pH 8.0), 0.5 μmoles of DPN, 5 μmoles of GSA, and 0.1 ml. of the enzyme solution. The total volume was made up to 3 ml. with distilled water. The reaction mixture was preincubated at 25°C for 5 minutes and the reaction was initiated by addition of GSA. Activity was assayed by measuring the increase of optical density at 340 mμ in a "Hitachi" spectrophotometer at 25°C.

The activity was expressed as the increase in optical density at 340 mμ in one minute. Protein was measured with Folin's reagents (*4*).

Materials—DPN and TPN were obtained from Sigma Chemical Co. Ethyl esters of the semialdehydes of adipic, glutaric, and succinic acid were kindly synthesized by Dr. T. Okuda of this Institute. In order to obtain free semialdehydes, 1000 μmoles of each ester in 5 ml. of distilled water were refluxed for 30 minutes in a boiling water bath with 1 g. of Amberlite IR 120 (H^+ form). The clear supernatant was neutralized and made up to 10 ml. with distilled water.

The potassium salt of β,β'-diethoxypropionate was kindly given by Dr. O. Hayaishi of Kyoto University. One hundred μmoles of malonic semialdehyde were prepared by dissolving this salt in 1 ml. of distilled water and adjust the pH to 2.0 with sulfuric acid. The mixture was stood for one hour at 48°C and then neutralized.

RESULTS

Purification of Dehydrogenase—Five g. of the acetone powder were homogenized with 200 ml. of 0.01 *M* phosphate buffer (pH 7.4). The mixture was allowed to stand overnight in a refrigerator. The homogenate was centrifuged and the precipitate was discarded. One g. of protamine sulfate in 5 ml. of distilled water was slowly added to the supernatant (Extract) with mechanical stirring. The pH of the solution was adjusted to 7.4. After 10 minutes the solution was centrifuged and the clear supernatant (Protamine treatment) was fractionated with ammonium sulfate. For ammonium sulfate fractionation, appropriate amounts of saturated ammonium sulfate solution of pH 7.4 were added and the mixture stirred for 30 minutes and then centrifuged. The fraction precipitating be-

* The following abbreviations have been used: GSA, glutaric semialdehyde; DPN, diphosphopyridine nucleotide: TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate; CoA, coenzyme A; PCMB, *p*-chloromercuribenzoic acid; EDTA, ethylenediaminetetracetic acid; Tris, tris (hydroxymethyl) aminomethane chloride.

tween 48 and 70 per cent saturation was dissolved in 20 ml. of 1×10^{-3} *M* phosphate buffer (pH 7.4) and dialized against 2 liters of the same buffer. After a few hours, the dialyzate (Ammonium sulfate fraction I) was mixed with Ca-phosphate gel in a protein to gel ratio of one to one. The pH was adjusted to 6.0 and the mixture stirred for 15 minutes. The gel was centrifuged and the supernatant was discarded. The gel fraction was eluted with 10 ml. of 0.1 *M* phosphate buffer (pH 7.0) for 15 minutes and centrifuged. The gel was again eluted with 5 ml. of 0.2 *M* phosphate buffer (pH 8.0). The activity was mainly eluted at pH 7.0, but occasionally some activity was found in the fraction eluted with buffer of pH 8.0. The eluate (Ca-gel treatment) was again fractionated with saturated ammonium sulfate solution and the fraction precipitating between 45 and 60 per cent saturation was dissolved in 7 ml. of the same buffer as used previously. This solution was dialyzed for 3 hours and used as the final enzyme solution (Ammonium sulfate fraction II). The above mentioned steps were all carried out at about 4°C and centrifugations were in each case at $15{,}000\times g$ for 10 minutes. A typical increase in specific activity and purification is shown in Table I. The specific activity increased about 30 fold and the yield was about 20 per cent.

TABLE I

Purification of GSA Dehydrogenase

	Total protein	Total activity	Specific activity
	mg.	Δ O.D./min.	Δ O.D./min./mg.
Extract	2355	92.0	0.039
Protamine treatment	1691	90.4	0.053
Ammonium sulfate fraction I	420	50.4	0.120
Ca-gel treatment	81	25.9	0.320
Ammonium sulfate fraction II	15	17.8	1.180

Reduction of DPN increased linearly with the incubation period and with the protein concentration as shown in Fig. 1 A and B.

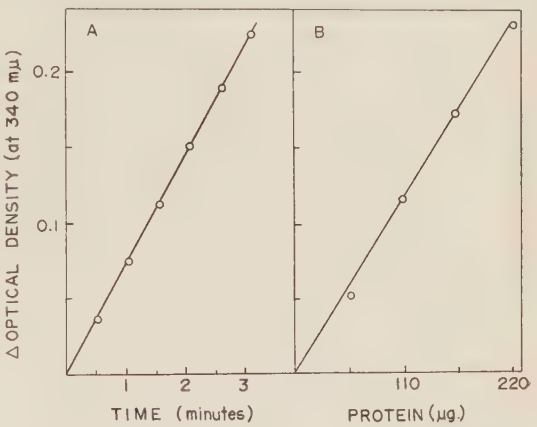

FIG. 1 A and 1 B. Relation of enzyme activity to incubation period and enzyme concentration.

pH Optimum—Fig. 2. shows that the pH optimum of the dehydrogenase is around pH 8.0. The decrease in activity on either side of this pH is almost symmetrical.

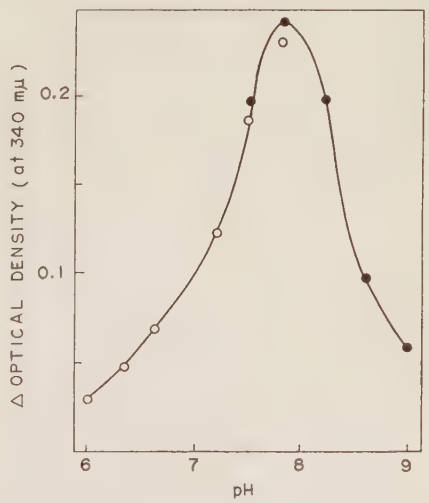

FIG. 2. pH optimum. The open circles indicate phosphate buffer and the solid ones Tris buffer.

Substrate Specificity—It was found that the enzyme is very specific for GSA and other

aldehydes tested—formaldehyde, glyoxalate, malonic and succinic semialdehydes—were not attacked, except for adipic semialdehyde, with which the enzyme showed about one third the activity observed with the same concentration of GSA.

Concentration of DPN and GSA—The optimal concentrations of DPN and GSA are shown in Figs. 3 and 4. The K_m values for these are $7.8\times10^{-6}M$ and $1.1\times10^{-3}M$ respectively. DPN was required even with the crude extract. TPN showed about 5 per cent of the activity of DPN.

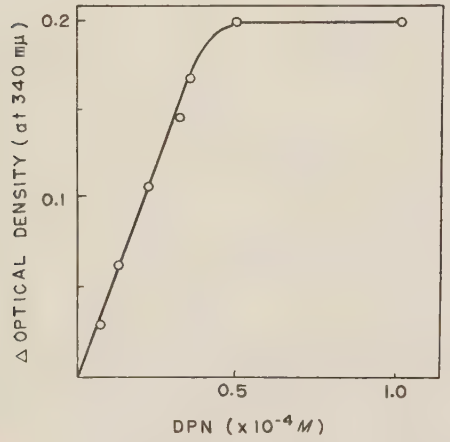

Fig. 3. Dehydrogenase activity with different concentrations of DPN.

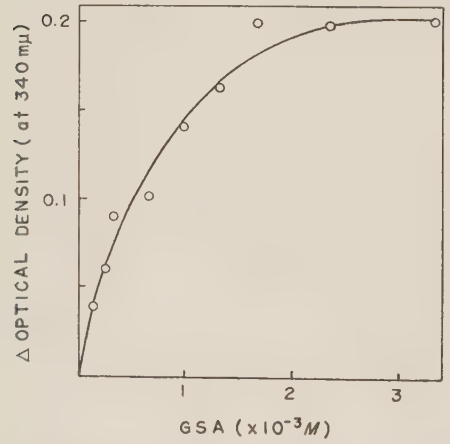

Fig. 4. Dehydrogenase activity with different concentrations of GSA.

Inhibitors and Reversibility of the Reaction—PCMB at a concentration of $3.3\times10^{-4}M$ completely inhibited the dehydrogenase, but arsenite and glutathione either together or separately had no effect. Co A with ATP was without effect. EDTA at a concentration of $3.3\times10^{-3}M$ stimulated the enzyme about 30 per cent.

There was no decrease in the optical density at 340 mμ when the enzyme was incubated with glutaric acid and reduced DPN at three different pHs namely, 6.3, 6.9 and 8.0.

Identification of the Product—The enzymic reaction was carried out as described in the "Assay Method" except for the addition of a somewhat larger amount of DPN (5 μmoles). After completion of the reaction, the ether extract of the reaction mixture was dried and applied to an Amberlite IR 112 column (1×90 cm.). The column was eluted with a mixture of acetone, methylene chloride and water (82:40:6 v/v). A large peak of acid was found in the eluate by titration with 0.005 *N* NaOH in the fractions between 15 and 24 ml. of the effluent and its location coincided with that of a sample of authentic glutaric acid, which had been tested previously on the same column. The substance in the acid peak was collected and chromatographed in three different solvents (*20*, *21*, *22*). This acid, located by spraying the papers with bromocresol green, showed the same R_f values as a sample of authentic glutaric acid.

DISCUSSION

The formation of glutaric acid from lysine was first suggested by Ringer *et al.* (*5*). Borsook *et al.* (*6*) using C^{14}-lysine with a homogenate of guinea pig liver, found that glutaric acid is indeed derived from lysine. Rothstein and Miller showed that in rats lysine was converted to glutaric acid via pipecolic acid (*7*).

A series of papers from this laboratory have reported that in *Pseudomonas* bacteria also DL-lysine was metabolized to GSA through δ-aminovaleric acid, (*1*, *2*, *8*, *9*). The present

paper shows that this GSA is oxidized to glutaric acid by a GSA dehydrogenase. DPN is probably the cofactor for this enzyme.

Analogous reactions have already been reported for succinic and malonic dehydrogenase by Jacoby *et al.* (*10 11*). The GSA dehydrogenase reported here differs from these enzymes reported by Jacoby, since neither succinic semialdehyde nor malonic semialdehyde can act as substrates for the GSA dehydrogenase, either in the presence or in the absence of CoA and ATP.

Stoppani and Millstein (*12*) and Jacoby (*13*) proposed that the thiol group of aldehyde dehydrogenases is essential for their activity. The finding that PCMB strongly inhibits GSA dehydrogenase suggests that the thiol group is playing an essential role in the formation of glutaric acid.

The formation of glutaric acid from tryptophan has recently been reported by Gholson *et al.* (*14*). The further metabolism of glutaric acid is still not clear. Rothstein and Miller proposed the direct conversion of this acid to α-ketoglutarate by α-oxidation (*15*). Recently Hobbs and Koeppe questioned this pathway and proposed that α-ketoglutarate was formed through acetate and the tricarboxylic acid cycle (*16*).

Using dog skeletal muscle, Stern *et al.* found that glutarate was activated by forming glutaryl-CoA (*17*). Recently we also found that glutarate was activated in the presence of ATP, CoA and Mg^{++} by the same strain of *Pseudomonas* bacteria as used in the present work (*18*). Nishizuka *et al.* also found that in *Pseudomonas flourescens* glutarate was converted to acetyl-CoA via glutaryl-CoA (*19*).

These results suggest that glutarate is probably metabolized via its thiol ester.

SUMMARY

1. A dehydrogenase was purified from *Pseudomonas* bacteria. This enzyme catalyzes the oxidation of glutaric semialdehyde in the presence of DPN.

2. The thiol group of the enzyme was shown to be essential for enzyme activity. The substrate specificity was found to be very high.

3. Glutaric acid was found to be the end product.

The authors wish to express their gratitude to Dr. M. Suda for his continuous support and advice in this work.

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The Journal of Biochemistry, Vol. 49, No. 2, 1961

Studies on Acetic Acid Bacteria

III. Purification and Properties of Coenzyme-Independent Aldehyde Dehydrogenase*

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(Received for publication, October 12, 1960)

Using resting cells and a cell free preparation, Tanenbaum (*1*) reported that *Acetobacter peroxydans* possesses alcohol and acetaldehyde dehydrogenases. Both of these may be TPN-dependent enzymes. In a previous paper (*2*), a procedure for the purification of TPN-dependent aldehyde dehydrogenase from *Acetobacter* sp., and its properties have been reported. A study of the intracellular distribution of the enzymes related to alcohol oxidation showed that this micro-organism might have another kind of aldehyde dehydrogenase which reduces ferricyanide in the presence of acetaldehyde, and which does not require either TPN or DPN. Furthermore, it was noticed that the dye-reducing enzyme could be more or less water-solubilized when certain conditions were used for its extraction.

The present paper describes the purification of the aldehyde-dye reducing enzyme from *Acetobacter* sp. and the properties of the purified enzyme. It was found that the enzyme was coenzyme-independent and differed from the TPN-dependent aldehyde dehydrogenase in several other properties.

MATERIALS AND METHODS

The strain of acetic acid bacteria used and the growth conditions were as described in the previous reports (*2*, *3*). The grown cells were dried with acetone by the conventional method. The resulting powder could be stored for at least one month in the cold (3–4°C) in a dry state without a noticeable loss of acetaldehyde-ferricyanide reducing activity.

* This work was done in the laboratory of Prof. K. Okunuki, Department of Biology, Faculty of Science, University of Osaka, Osaka, Japan.

Reduction of ferricyanide by aldehydes and alcohols was assayed by measuring the decrease in optical density at 400 mμ in a system with the following components: 0.1 ml. of enzyme solution, 3.0 ml. of 0.04 *M* buffer, 0.1 ml. of 0.2 *M* substrate, 0.1 ml. of 0.02 *M* potassium ferricyanide and water to a total volume of 3.4 ml.. Citrate-phosphate buffer, pH 7.0, was used for experiments on aldehyde oxidation and citrate buffer, pH 3.0, for those on alcohol oxidation, unless otherwise indicated. Reduction of TPN by aldehyde was assayed by following the increment in optical density at 340 mμ in a system with the following components: 0.1 ml. of enzyme solution, 3.0 ml. of 0.04 *M* phosphate buffer of pH 9.0, 0.1 ml. of 6.0 m*M* TPN, 0.1 ml. of 0.2 *M* acetaldehyde and water to a total volume of 3.4 ml..

Spectrophotometric assays were carried out at room temperature (24°C) with a Shimadzu photoelectric spectrophotometer, type QB-50, using cuvettes of one cm. optical path. The enzyme activity was calculated from the initial linear reaction rate, and expressed as $-\Delta E^{\text{Ferri.}}_{400m\mu}$ for the reduction of ferricyanide and as $\Delta E^{\text{TPN}}_{340m\mu}$ for the reduction of TPN. One ferricyanide unit and one TPN unit of enzyme were defined as the concentration of enzyme causing an initial $-\Delta E^{\text{Ferri.}}_{400m\mu}$ and $\Delta E^{\text{TPN}}_{340m\mu}$ of 0.001 per minute, respectively. The protein concentration of samples was expressed by the optical density at 280 mμ, $E_{280m\mu}$. Specific enzyme activity was expressed as $-\Delta E^{\text{Ferri.}}_{400m\mu}/E_{280m\mu}$/minute and as $\Delta E^{\text{TPN}}_{340m\mu}/E_{280m\mu}$/minute. In some experiments, enzymatic reduction of oxidation-reduction dyes was studied anaerobically at 30°C in Thunberg tubes according to the conventional method.

Crystalline cytochrome c was prepared from bovine heart muscle according to the method of Bodo (*4*) using the slight modification of Yamanaka *et al.* (*5*). TPN was purchased from Sigma Chemical Co., Missouri, U. S. A. (90 per cent pure) and aluminium oxide for chromatography from Wako Pure Chemical Indust-

rial Co., Ltd., Osaka. The aluminium oxide was reactivated by treatment with acid and alkali (*6*).

EXPERIMENTAL AND RESULTS

Purification of the Acetaldehyde-Ferricyanide Reducing Enzyme—Acetone-dried cells of *Acetobacter* sp. (75 g.) were mixed with 1.500 ml. of 0.1 *M* citrate-phosphate buffer (pH 3.0) using a homomixer (15,000 r. p. m.) at room temperature (24°C) without any cooling devise. Five hundred ml. aliquots of the cell suspension were mixed using a 2,000 ml. vessel. If the aliquots were mixed for less than 10 minutes, little enzyme could be extracted. Under the above experimental conditions, a mixing time of 10 minutes or a little more was suitable for the extraction. The mixture was centrifuged (10,000×*g* for 10 minutes). The cellular debris was re-extracted with 750 ml. of buffer in the same manner. The second extract had one third as much acetaldehyde-ferricyanide reducing activity as the first. The two extracts were combined. The yellowish brown mixture (1,880 ml.) contained the TPN-dependent aldehyde dehydrogenase (*2*), the acetaldehyde-ferricyanide reducing enzyme (coenzyme-independent aldehyde dehydrogenase) and an ethanol-ferricyanide reducing enzyme (alcohol-cytochrome-553 reductase (*7*)), and showed a cytochrome-like absorption band at 553 mμ (*7*).

The mixture was passed through an aluminium oxide column (15×5 cm.) which had been equilibrated with 0.1 *M* citrate-phosphate buffer (pH 3.0). The flow rate was 1,500 ml. per hour. The three enzymes and the hemeprotein showing an absorption band at 553 mμ were completely absorbed on the

Scheme I

Purification of acetaldehyde-ferricyanide reducing enzyme from *Acetobacter* sp.

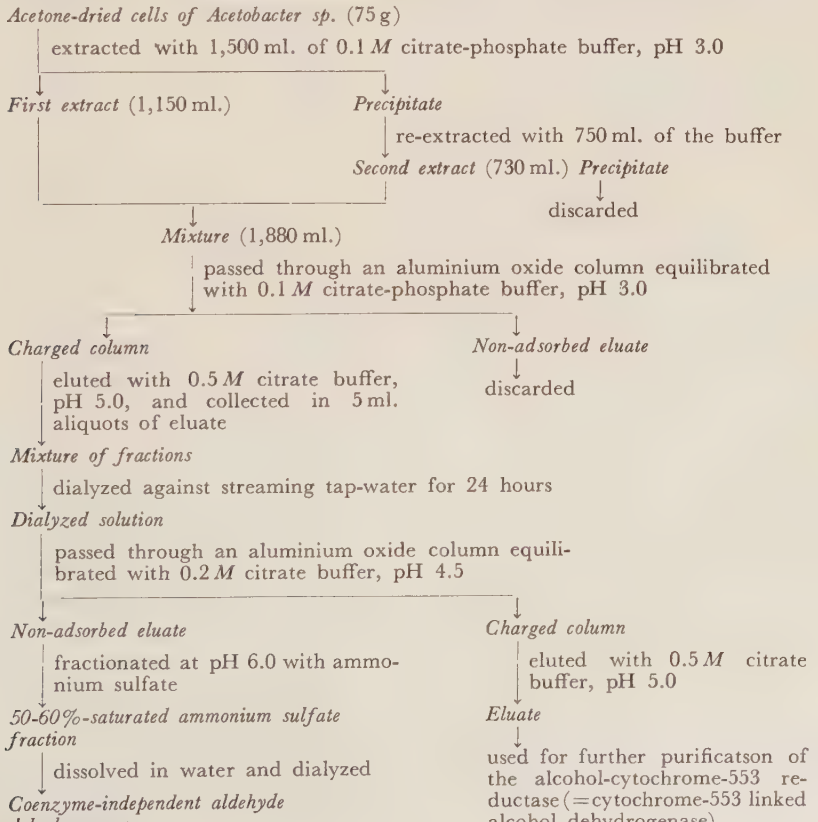

TABLE I

Summary of the Purification of the Acetaldehyde-Ferricyanide Reducing Enzyme

Purification step	Total units ($-\Delta E^{Ferri.}_{400m\mu}$/min.)	Specific activity ($-\Delta E^{Ferri.}_{400m\mu}/E_{280m\mu}$/min.)	Yield (%)	Purity
Mixture of 1st and 2nd extracts	2,150,000	400	(100)	(1)
Eluate from pH 3-aluminium oxide column	1,200,000	3,000	56	7.5
Dialyzed eluate	800,000	2,600	37	6.5
Non-adsorbed eluate from pH 4.5-aluminium oxide column	670,000	7,500	31	19
Ammonium sulfate fractionation, 50–65% saturation	435,000	13,400	20	32

column and then they were eluted with 0.5 *M* citrate buffer (pH 5.0). The eluate (100 ml.) containing the enzymes and the hemeprotein was dialyzed against streaming tap water at 10–15°C for 24 hours. During the dialysis, the TPN-dependent aldehyde dehydrogenase (*2*) was completely inactivated, while the acetaldehyde-ferricyanide reducing enzyme and ethanol-ferricyanide reducing enzyme scarcely lost their activity. The dialyzed solution was passed through an alminium oxide column (15×5 cm. diameter) which had been equilibrated with 0.2 *M* citrate buffer (5.0). The ethanol-ferricyanide reducing enzyme and the hemeprotein were adsorbed on the column but the acetaldehyde-ferricyanide reducing enzyme was not. The eluate containing the latter enzyme was fractionated between 50 and 60 per cent saturation with ammonium sulfate at approximately pH 6 and the precipitate was collected. There was a 32 per cent yield, and the purity was 30 times as high as that of the extract.

The purification procedure is shown in Scheme I and the yield and purity achieved at each step are given in Table I.

Experiments were carried out with the enzyme at the final stage of purification.

General Properties of the Enzyme

Electron Acceptors—In the presence of acetaldehyde, the purified acetaldehyde-ferricyanide reducing enzyme could reduce various oxidation-reduction dyes such as 2,6-dichlorophenol-indophenol, thionine and methylene blue (Table II). In the presence of bicarbonate (pH 7.0) and methylene blue, evolution of carbon dioxide could be manometrically demonstrated at 30°C during the oxidation of acetaldehyde to acetic acid if a high concentration of the enzyme was used.

TABLE II

Electron Acceptors of the Acetaldehyde-Ferricyanide Reducing Enzyme

Electron acceptor	Conc. of enzyme ($E_{280m\mu}$)	Time for decolorization (mins.)
2,6-Dichlorophenol-indophenol	3×10^{-5}	~0
Ferricyanide	3×10^{-4}	5
Thionine	3×10^{-3}	42
Methylene blue	3×10^{-3}	600
	3×10^{-2}	15
Indigo tetrasulfonate	3×10^{-2}	>720
Nile blue	3×10^{-2}	>720
Phenosalfanine	3×10^{-2}	>720

Effect of pH on the Enzyme Activity—Of the four buffers tested, the acetaldehyde-ferricyanide reducing enzyme showed the highest activity in citrate-phosphate buffer, its pH optimum being at pH 7.0 (Fig. 1). The optimal pH varied slightly in the other buffers, between pH 7.0 and 7.8.

Effect of Temperature and pH on the Stability of the Enzyme—The acetaldehyde-ferricyanide reducing enzyme was fairly stable over a broad pH-range at low temperature, being most stable at pH 5.0 (Fig. 2).

If dissolved in citrate-phosphate buffer

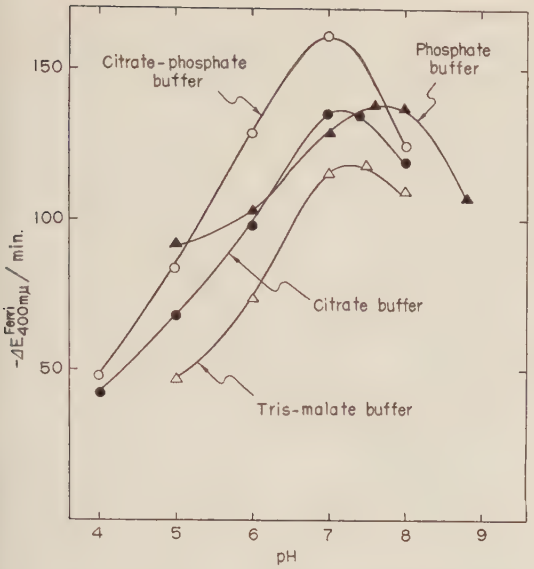

Fig. 1. Effect of pH on the acetaldehyde-ferricyanide reducing activity.

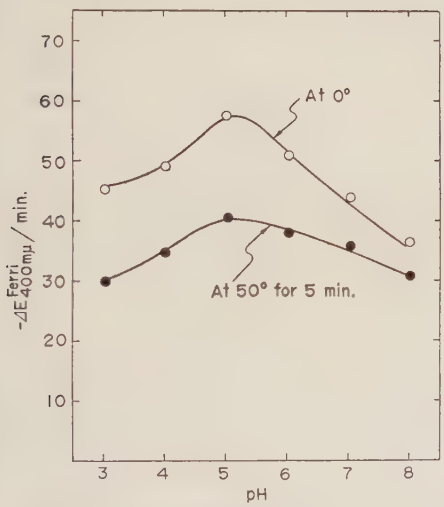

Fig. 2. Effect of pH and heat on the stability of the acetaldehyde-ferricyanide reducing enzyme.

(pH 5.0), the enzyme could be stored at 3–4°C for a long time without a notable loss of activity. However the enzyme was rapidly inactivated at temperatures above 40°C.

Substrate Specificity—The aldehyde-ferricyanide reducing enzyme showed a very broad substrate specificity (Table III). Of the aldehyde tested, the enzyme had strongest affinity for acetaldehyde, propionaldehyde, *n*-butylaldehyde and crotonaldehyde. Unlike the TPN-dependent aldehyde dehydrogenase (*2*), the present enzyme rapidly oxidized crotonaldehyde and benzaldehyde. The K_m for acetaldehyde was 8.7×10^{-5} moles/liter (Fig. 3), and for ferricyanide 8.2×10^{-4} moles/liter (Fig. 4).

Table III

Substrate Specificity of the Aldehyde-Ferricyanide Reducing Enzyme

Substrate	Aldehyde-ferricyanide reducing activity	
	$(-\Delta E^{Ferri.}_{400m\mu}$/min.)	Time for decolorization (mins.)
Formaldehyde	<0.008	73
Acetaldehyde	0.118	4
Propionaldehyde	0.108	4
n-Butylaldehyde	0.100	4
n-Valeraldehyde[1)]	—	5
n-Capronaldehyde[1)]	—	5
n-Oenanthaldehyde[1)]	—	5
n-Caprylaldehyde[1)]	—	6
Crotonaldehyde	0.104	4
Benzaldehyde[1)]	—	6

1) Added as an emulsion.

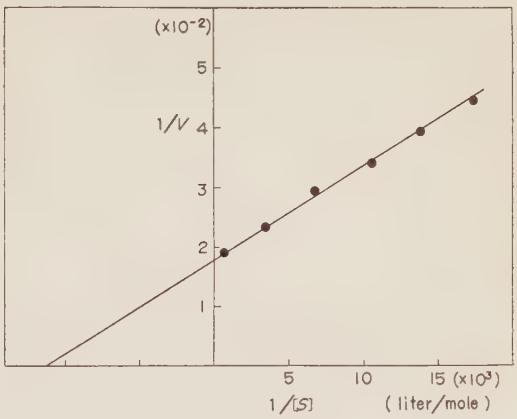

Fig. 3. 1/V-1/[S] curve for the acetaldehyde-ferricyanide reducing enzyme. Assayed in the presence of 5.8×10^{-4} *M* potassium ferricyanide.

Effect of Metal Ions on the Enzyme Activity—None of the metal tested activated the acetaldehyde-ferricyanide reducing enzyme. Most of those tested were more or less inhibitory,

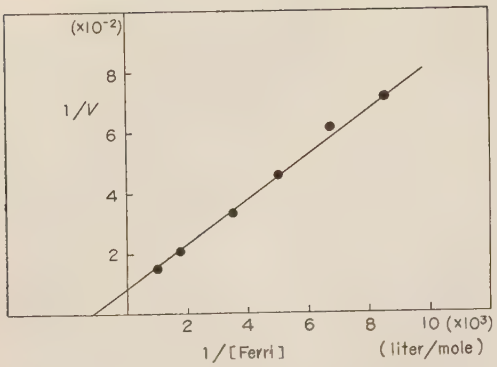

FIG. 4. 1/V-1/[Ferri.] curve for the acetaldehyde-ferricyanide reducing enzyme. Assayed in the presence of 5.8×10^{-3} *M* acetaldehyde.

Ca^{++} and Mn^{++} having the greatest inhibition (Table IV).

TABLE IV

Effect of Ions on Acetaldehyde-Ferricyanide Reducing Activity

Ion	Inhibition (%)	
	Conc. of ion added	
	$6 \times 10^{-4}M$	$3 \times 10^{-3}M$
NH_4^+	8	18
K^+	0	14
Mg^{++}	4	14
Ca^{++}	24	—
Zn^{++}	4	—
Mn^{++}	32	—
Co^{++}	7	—
Cu^{++}	0	—
Fe^{+++}	0	—
SO_4^{--}	9	22

Effect of Inhibitors on the Enzyme Activity—Of the reagents tested, only *p*-chloromercuribenzoate inhibited the acetaldehyde-ferricyanide reducing enzyme activity strongly (Table V). The enzyme was only slightly inhibited by hydroxylamine (Table VI) which had been found to be a strong inhibitor of the TPN-dependent aldehyde dehydrogenase (*2*).

TABLE V

Effect of Inhibitors on the Acetaldehyde-Ferricyanide Reducing Activity

Reagent	Inhibition (%)	
	Conc. of reagent added	
	$6 \times 10^{-4}M$	$3 \times 10^{-3}M$
Sulfhydryl reagent:		
Monoiodoacetate	0	6
p-Chloromercuribenzoate	44	100
Arsenite	12	11
Carbonyl reagent:		
Hydroxylamine	15	17
Semicarbazide	11	21
Hydrogen sulfite	2	9
Metal-chelating reagent:		
Cyanide	0	0
Ethylenediamine tetra-acetate	2	12
o-Phenanthroline	21	37
Fluoride	11	17
Pyro-phosphate	4	9

TABLE VI

Effect of Hydroxylamine on the Acetaldehyde-Ferricyanide Reducing Activity

Components preincubated	Final conc. of hydroxylamine (*M*)	Inhibition (%)
Enzyme	—	(0)
Enzyme	3×10^{-3}	17
Enzyme+hydroxylamine	3×10^{-3}	19
Acetaldehyde+hydroxylamine	3×10^{-3}	2

DISCUSSION

Acetobacter sp. has been used for vinegar manufacture because it can form much acetic acid. Two aldehyde dehydrogenases have been extracted from this bacteria and separately purified. In the presence of aldehydes, one of them catalyzes the reduction of TPN but not DPN, while the other does not reduce either coenzyme but catalyzes the reduction of dyes such as ferricyanide. These two enzymes differ in pH optimum, activation by

metal ions (especially Mg^{++} and Mn^{++}), substrate specificity (especially with regard to crotonaldehyde and benzaldehyde) and in the effect on them of some inhibitors (especially hydroxylamine). The enzyme previously named the aldehyde-ferricyanide reducing enzyme, seems more suitably designated as the "coenzyme-independent aldehyde dehydrogenase (*Acetobacter*)".

Gordon *et al.* (*8*) have purified an aldehyde oxidase from pig liver and found that it catalyzes the anaerobic reduction of methylene blue by aldehydes regardless of the presence or absence of coenzymes. Mahler *et al.* (*9*) have found that this enzyme can reduce 2,6-dichlorophenol-indophenol and mammalian heart cytochrome c in the presence of aldehydes. The aldehyde oxidase is an enzyme binding flavine adenine dinucleotide, which showes an atypical flavoprotein spectrum with maxima at 278, 350 (diffuse), and 405 mμ, and shoulders at 450, 530 and 630 mμ. On reduction with aldehydes or dithionite, the peak at 350 mμ disappears, the peak at 405 mμ is lowered, the shoulder at 450 mμ disappears, and peaks appear at 560 mμ and 620 mμ. Furthermore, it has been found that enzymic reduction of cytochrome c occurs only in the presence of Mo^{++}.

The coenzyme-independent aldehyde dehydrogenase (*Acetobacter*) rapidly reduces ferricyanide, an uni-electron acceptor, and 2,6-dichlorophenol-indophenol, a di-electron acceptor, in the presence of aldehydes. Therefore, the dehydrogenase may be similar to Gordon *et al.*'s enzyme. The enzyme reaction can be expressed by the equations:

$$R\cdot CHO + H_2O \longrightarrow R\cdot COOH + 2H$$
$$2H \longrightarrow 2H^+ + 2e^-$$

Substrate specificities of the two enzymes are also similar, but the coenzyme-independent aldehyde dehydrogenase (*Acetobacter*) oxidizes acetaldehyde, propionaldehyde, *n*-butylaldehyde and crotonaldehyde at almost the same rate, while the aldehyde oxidase (pig liver) oxidizes acetaldehyde, propionaldehyde, *n*-butylaldehyde, crotonaldehyde, benzaldehyde and glycollicaldehyde at rates of 100:17:13:66:25:8, respectively. Therefore the two enzymes clearly differ in their properties.

SUMMARY

It was found that *Acetobacter* sp. contains a coenzyme-independent aldehyde dehydrogenase beside a TPN-dependent one. The former was purified thirty-fold and its general properties were examined.

1. The enzyme reduced both uni- and di-electron acceptors such as ferricyanide, 2,6-dichlorophenol-indophenol and methylene blue in the presence of acetaldehyde, but not TPN and DPN.

2. Its pH optimum was at pH 7.0 in citrate-phosphate buffer. It was fairly stable at pH 5.0 at a low temperature, but not at above 40°C.

3. Its substrate specificity was very broad, and it rapidly oxidized acetaldehyde, propionaldehyde, *n*-butylaldehyde and crotonaldehyde at almost the same rate. Its K_m for acetaldehyde was 8.7×10^{-5} moles/liter and for ferricyanide was 8.2×10^{-4} moles/liter.

4. Its activity was strongly inhibited by *p*-chloromercuribenzoate, but it was little affected by hydroxylamine.

The reaction mechanism of the enzyme was discussed.

The author would like to express his sincere thanks to Prof. K. Okunuki, Department of Biology, Faculty of Science, University of Osaka, Osaka, for his valuable guidance during this study, and to Miss. A. Inoue for her technical assistance.

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Studies on Conjugation of S^{35}-Sulfate with Phenolic Compounds

VI. Sulfate Conjugation of Xanthurentic Acid in Rat Liver

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It has been reported by Kotake *et al.* (*1*), Rotstein *et al.* (*2*), and Baglioni *et al.* (*3*) that xanthurenic acid (XA) is excreted in the urine of animals as conjugates. Using XA-C^{14}, Rotstein *et al.* (*2*) have shown that no degradation of XA occurred in rats or rabbits but that in rats it is metabolised into two kinds of conjugate containing glucuronic acid and serine. In rabbits XA is metabolised to a conjugate of sulfuric acid. Baglioni *et al.* (*3*) have detected two metabolites, of which one was a conjugate of XA with sulfate and glycine.

In the present study XA is reported to be conjugated with sulfate in the liver or kidney of the rat.

EXPERIMENTAL AND RESULTS

The incubation of XA with rat liver slices or with the supernatant of rat liver homogenate was conducted as described previously (*4*) with a modification at the deproteinization step, where three volumes of ethanol were used instead of trichloroacetic acid solution. Ascending paper chromatography was carried out in *n*-butanol, acetic acid, water 4:1:1 (BAW), methanol, *n*-butanol, benzene, water 2:1:1:1 (MBBW) and MBBW containing 1% of 15 *N* ammonia solution (NH_3-MBBW) (*5*). The diazo reaction was carried out as described previously, using sulfanilic acid (*6*).

Chemical Synthesis of Sulfate Conjugates of XA—Xanthurenic acid (10 mg.) was dissolved in pyridine (0.75 ml.) at 0°C. To this a mixture of chlorosulfonic acid (0.05 ml.) and chloroform (0.125 ml.) was added slowly. The mixture was stirred for two hours. After standing overnight the solution was neutralized with potassium hydroxide (2 *N*), extracted with ether and the aqueous phase evaporated to a small volume. This was examined on paper chromatograms and two sulfate conjugates of XA were found after examining the fluorescence under ultraviolet light. One of the conjugates showed a strong blue fluorescence with the R_f value of 0.26 run in MBBW, 0.30 in NH_3-MBBW, and 0.13 in BAW. The freshly chromatographed spot showed a very faint red diazo reaction but after several days it turned out to be definitely positive in diazo reaction. After standing several days it was rechromatographed and showed a spot of XA and the original spot. It was rechromatographed after it was sprayed with diazo reagents and showed the original fluorescent spot and another red spot which coincided with the diazotized XA. Summarizing the results, the substance at R_f 0.26 run in MBBW is negative in diazo reaction

and easily hydrolysable on standing to produce XA which is positive in the diazo reaction. It has been reported that the hydroxyl group at the 8-position of XA should be free for a positive diazo reaction (*1*, *2*, *5*). From these observations the substance was concluded to be conjugated at the 8 position and it appeared to be the sulfate conjugate of XA at the 8 position (8-OH sulfate).

There appeared another minor fluorescent spot on the starting line with MBBW and NH_3-MBBW. It was negative in diazo reaction but produced XA by hydrolysis with hydrogen chloride fumes for one hour. The characterisation of this substance will be referred to later.

Experiment with the Supernatant of Rat Liver Homogenates—Fifty μg. of XA, about 1 μc. of radioactive sulfate, and 3 mg. of potassium ATP were incubated with 0.1 ml. of the supernatant of rat liver homogenate which had been made with 2 volumes of Ringer solution devoid of sulfate. The reaction mixture was deproteinized, evaporated to a small volume and chromatographed. The paper showed a strong fluorescent spot at 0.26, run in MBBW. It coincided with the radioautographical spot. The nature of the spot was similar to that of 8-OH sulfate. This spot was chromatographed successively with MBBW, NH_3-MBBW and BAW; the ninhydrin reaction was negative. The original spot was cut out and eluted with water. This eluate was mixed with the eluate of non-labelled 8-OH sulfate, evaporated to a small volume and paper chromatographed. The pattern of radioautography matched with the spots of 8-OH sulfate. There appeared also a small quantity of inorganic sulfate and XA which were thought to be the decomposition products. A small quantity of 8-OH sulfate was detected by the method described above in the control experiment with the supernatant of rat liver homogenate without adding any XA. This shows that 8-OH sulfate is one of the normal metabolites of tryptophan in rat liver.

There appeared two other minor sulfate conjugates. One did not move in MBBW and NH_3-MBBW and it showed common characteristics with the one synthesized and showed negative diazo reaction. This was eluted with water and hydrolyzed in *N* HCl at 100°C for 30 minutes. The solution was neutralized with *N*-NaOH and added to diazo reagents. The colour was determined by Beckmann DU at the wavelength of 502.5 mμ. Radioactivity was measured by gas flow counter after the sulfate was precipitated with barium chloride solution. The ratio of XA to radioactivity was calculated to be about the half of that with 8-OH sulfate from the same paper. From these results this substance was though to be XA conjugated with two sulfates. The other substance appeared at 0.13 in MBBW and 0.39 in NH_3-MBBW. As the quantity was small, identification was not possible.

Experiments with Liver or Kidney Slices of the Rat—Incubating XA with rat liver slices in Ringer solution devoid of sulfate in oxygen, there appeared after paper chromatography a substance which was radioactive showing similar R_f values in different solvents with 8-OH sulfate. A small quantity of the substance was found in the control experiment as shown in the case with the supernatant.

There appeared another spot at the R_f value of 0.22 in MBBW, 0.12 in BAW and 0.19 in NH_3-MBBW. This substance showed strong fluorescence but was negative in diazo reaction and did not contain sulfate as shown by examination by radioautography. It showed positive glucuronic acid reaction by naphthoresorcinol reagent. There was another minor conjugate which contained sulfate at 0.13 run in MBBW and 0.39 in NH_3-MBBW. This was similar to the spot observed in the experiment with the supernatant.

The reaction mixture of XA (5 mg.) and liver slices (approx. 2.5 g.) from male adult rats in Ringer phosphate solution (30 ml.) was adjusted to pH 4 with acetic acid and centrifuged for 20 minutes at 600 *g*. The supernatant was treated with activated charcoal (0.5 g.) and the charcoal filtered off and washed with water. The material obtained by the subsequent elution of the charcoal with

methanol containing 0.2% (v/v) of aq. NH_3 solution (sp. gr. 0.88) and evaporation under reduced pressure was examined on paper chromatograms. The conjugate at the R_f value of 0.13 run in BAW was not observed under ultraviolet light, although the original reaction mixture showed very strong fluorescence at this position. This shows that the sulfate conjugate of XA at the 8 position is unable to be concentrated by the activated charcoal technique.

The results of the experiment with rat kidney slices were similar to that with the liver slices, although the quantities of conjugates formed were smaller.

Radioactive 8-OH sulfate was extracted with Ringer solution and incubated with rat liver slices; no other sulfate conjugate was observed except the original substance, inorganic sulfate and xanthurenic acid on examination of fluorescence and radioautography.

Sulfate Conjugates in the Urine of the Rat Fed with Xanthurenic Acid—Rats were fed with 10 mg. of XA daily and about 50 μc. of radioactive sulfate was injceted subcutaneously. The urine excreted within the next 24 hour period was collected. This was applied to paper chromatograms and compared with that of the control animal. Two substances at the R_f values of B and D substances, which have been described in the paper of Rotstein *et al.* (*2*), were observed, but only a very small quantity of 8-OH sulfate was found. Other minor sulfate conjugate was found at 0.40 run in MBBW and 0.14 in BAW. This showed faint fluorescence under ultraviolet light, but was negative in diazo reaction. It became positive in the reaction only after the paper was subjected to hydrolysis in hydrogen chloride fumes. It showed very faint radioactivity comparing with that of indican on the same paper chromatogram. The spot of indican was easily detectable from the purple black colour when the paper was submitted to the fumes of hydrogen chloride, and from the colour reaction of the eluate using Jolles reaction (*7*).

Examination of the Bile of the Rat Injected with XA—A rat was operated on to obtain bile according to the method of Boyland, Ramsay and Sims (*8*). The rat was injected with 50 mg. of XA intraperitoneally and the bile was collected for 24 hours. The bile was examined by paper chromatography and it was not possible to detect fluorescence at the position of 8-OH sulfate. The rat received another injection of 50 mg. of XA and the bile showed weak fluorescence at the position of 8-OH sulfate.

The bile obtained after administering XA (100 mg.) intraperitoneally was deproteinized with three volumes of ethanol. The supernatant was evaporated under reduced pressure to a small volume and this solution was applied to the base lines of large sheets of Whatman 3*M* chromatography paper, which was then developed with BAW. The areas which showed as fluorescent bands under ultra violet light were eluted with methanol containing 5% ammonia (sp. gr. 88) and the eluates were then evaporated to small volume under reduced pressure. These fractions were examined by paper chromatography using MBBW, BAW, 20% (w/v) potassium chloride solution and aq. 2% acetic acid, *iso*-propanol (3:7, by vol.) as described earlier (*2*, *3*, *10*).

The major metabolite appeared as a bright fluorescent spot which could not be distinguished from authentic xanthurenic acid-8-methyl ether in either of the four solvent systems. Xanthurenic acid was also present in these fractions together with other fluorescent compounds. A comparison of the R_f values of these compounds with those reported in the literature (*2*, *3*, *10*) indicated the presence of a compound in which xanthurenic acid is conjugated with glycine. Evidence was also obtained for the presence of the glucuronic acid conjugate and the sulfuric acid conjugate of this glycine derivative as described by Baglioni *et al.* (*3*).

DISCUSSION

The data presented in this paper indicate that xanthurenic acid is conjugated with sulfate *in vitro* experiments with the liver of

the rat. Rotstein *et al.* (*2*) reported that XA-C^{14} was conjugated with glucuronic acid and serine but not with sulfate in the urine of rats fed with XA. Baglioni *et al.* (*3*) reported that XA was conjugated with sulfate, at 8 position, and glycine after intraperitoneal administration of XA in amounts of 300 mg./Kg. body weight. The difference between the two papers might have been because of the quantity of XA or species difference of the experimental animals. In the present study the quantity of S^{35}-sulfate conjugates was small compared with other metabolites in *in vivo* experiments.

The main sulfate conjugate of XA in rat liver was concluded to be conjugated at the hydroxyl radical at the 8 position. This substance appeared in the control experiments, to which XA was not added, using the supernatant of rat liver homogenate or rat liver slices. This substance was very abundant in *in vitro* experiments using XA but only a trace was recovered from the urine of the rats fed with XA, though indican was found in considerable amount. Rotstein *et al.* (*2*) found that XA-C^{14} was excreted completely in the urine of rats when injected intraperitoneally. In the present experiments it has been proved that sulfate conjugates of XA were only slightly excreted in the bile of rats injected with XA. This shows that two categories of sulfate conjugates are formed in animal body, one group of conjugates appear in the urine like indican or phenyl sulfate, and the others are formed in the liver but are difficult to detect in the urine. The latter group includes the sulfate conjugates of XA, pentachlorophenol and 2,4-dinitrophenol which are easily decomposed on standing and are thought to have high potential (unpublished data). They are able to be chromatographed using neutral or alkaline solvents but are easily decomposed with acidic solvents. That the urine of rats dosed with XA contained very little sulfate conjugated to XA indicates that the sulfate conjugated to XA had been decomposed in the body or transferred to other substances in the body. The sulfate might be able to be transferred to other substances *via* 3′,5′-diphosphoadenosine as shown by Gregor and Lipmann in the case from *p*-nitrophenyl sulfate to phenylsulfate (*9*).

SUMMARY

Xanthurenic acid has been shown to be readily conjugated with sulfate in the presence of rat liver or kidney slices as well as the supernatant of liver homogenate. The position of the conjugation of the main product was concluded to be at 8 position of xanthurenic acid. This substance was found without adding any xanthurenic acid in the control experiment. In an *in vivo* experiment, xanthurenic acid was found to be conjugated very little with sulfate in the urine of rats dosed with XA, as already reported by Rotstein.

We wish to thank Prof, E. Boyland and Dr. P. Sims for their kind interest and for providing an authentic specimen of xanthurenic acid-8-methyl ether. The biliary fistula was prepared at the Chester Beatty Research Institute by Mr. G. S. Ramsay, F. R. C. S., and Miss M. Biggs, to whom we express our sincere thanks.

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Action of Pancreatic Lipase in Homogeneous Solution

IV. Effect of Synthetic Food Colors on Pancreatic Lipase

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The effect of synthetic dyes on pancreatic lipase have been studied by a few investigators (*1*, *2*). However, any appreciable information on this subject has not been obtained as yet. Besides, the determination of lipase activity has been carried out with emulsions of many kinds of fatty acid esters in water. The disadvantage of such methods is that lipase action is examined in heterogeneous solution.

Therefore, in the preceding studies of this series (*3*, *4*) the behavior of pancreatic lipase towards nonionic surfactants (water-soluble higher fatty acid esters) as substrate in homogeneous solution was investigated, the activating power of bile salts, lecithin and cholesterol to the enzyme being reexamined under the condition.

The present paper deals with the inhibiting action of synthetic food colors, which are widely used industrially and domestically, on pancreatic lipase in homogeneous solution.

MATERIALS AND METHODS

Materials—The certified food colors which are officially permitted to apply to foods in Japan were used without further purification, but each of them was proved to be chromatographically pure (*5*). Water-soluble dyes were dissolved (directly) in water, but fat-soluble dyes were suspended in 1% lecithin solution, as in the case of cholesterol suspension, according to the method previously reported (*4*).

Substrate—A commercial product of polyoxyethylene sorbitan monolaurate (Nikkol TL-10)* was used as a substrate after purification by the method previously described (*3*).

Lipase Assay—Lipase action was estimated by the same manometric method at 37°C and pH 6.9 as mentioned previously (*3*).

Graphic Analysis of the Results—In the cases of competitive and noncompetitive inhibition, next two equations have been introduced by Schwimmer (*6*):

$$\frac{V_o}{V_i} = 1 + \frac{[I]}{K_i}\left(\frac{K_m}{K_m+[S]}\right) \qquad (1)$$

$$\frac{V_o}{V_i'} = 1 + \frac{[I]}{K_i} \qquad (2)$$

where $[S]$ and $[I]$ are the concentrations of the substrate and the inhibitor, K_m and K_i are the dissociation constant of the enzyme-substrate complex and the enzyme-inhibitor complex, V_o is the velocity of the reaction in the absence of inhibitor and V_i or V_i' is the velocity of the reaction in the presence of inhibitor. Equation (1) and (2) are used for competitive and noncompetitive inhibition, respectively.

In the case of competitive inhibition (equation (1)), when V_o/V_i is plotted against inhibitor concentration $[I]$, straight lines are obtained with unit intercept on the V_o/V_i axis and with slope dependent on $[S]$.

In the case of noncompetitive inhibition (equation (2)), plot of V_o/V_i' *vs.* $[I]$ gives a straight line with unit intercept and slope of $1/K_i$, independent on $[S]$. From the graph the value of K_i can be evaluated.

RESULTS AND DISCUSSION

Effect of Azo Dyes—Various azo dyes having a similar chemical constitution and an azo pyrazolone dye (Tartrazine) were tested for their inhibitory effects on lipase action. The results are shown in Fig. 1 and Table I.

In the case of Ponceau R, the value V_0/V_i' was plotted against $[I]$ as shown in Fig. 1. Thus, one straight line was obtained for all TL concentrations (5.0×10^{-2}, 2.5×10^{-2}, 1.25×10^{-2} *M*), and there was an intercept of

* TL is used for Nikkol TL-10.

1.0 on the ordinate. This means that the slope of the line is independent on the substrate concentration, and therefore the inhibition is noncompetitive. The value of K_i was estimated from the graph to be 1.5 mg. per ml. Exactly the same procedure was carried out with the other water-soluble azo dyes (Ponceau 3R, Ponceau SX, Orange 1, Sunset Yellow FCF and Tartrazine), and the values K_i for them are shown in Table I.

In the case of fat-soluble azo dyes (Orange SS, Yellow AB and Yellow OB), all these dyes had no effect on lipase action in 0.1% (w/v) solutions. But the effect of Oil Red XO (fat-soluble azo dye) on lipase action could not be measured, since good stable emulsion was not obtained from it.

Effect of Xanthene Dyes—The effects of 3 different xanthene dyes (Phloxine, Erythrosine and Eosine) on the hydrolysis of TL by lipase was examined under the conditions where azo dyes were tested. The results are summarized in Fig. 2 and Table I, which show that these dyes cause powerful noncompetitive inhibition on the lipase action. But Uranine (not food color) and KBr were observed to be without effect in the concentration of 0.2 per cent (w/v).

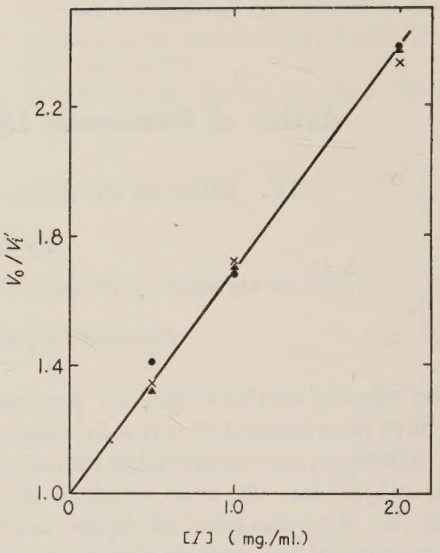

FIG. 1. Noncompetitive inhibition of pancreatic lipase by Ponceau R. A mixture consisting of enzyme solution* (0.2 ml.), buffer ($M/2$ $NaHCO_3$, 0.4 ml.) and water (usually 0.5 ml.) was incubated with the dye solution (0.4 ml.) under shaking for 20 minutes at 37°C, and then the substrate solution (0.5 ml.) was added to. The manometers were aerated with 100% CO_2. pH of the reaction mixture was 6.9. The determination of activity was carried out manometrically at 37°C for 10 minutes. The following concentrations of the substrate (Nikkol TL-10) were used: —●— 5.0×10^{-2} M; —×— 2.5×10^{-2} M; —▲— 1.25×10^{-2} M.

TABLE I

Effect of Food Colors on Pancreatic Lipase

The same conditions as shown in Fig. 1 were used.

FD&C	Common name	CI	Inhibition (%)[1]	K_i (mg./ml.)
Azo dye				
Red No. 1	Ponceau 3R	80	23	3.3
,, ,, 4	Ponceau SX	—	22	3.6
,, ,, 5	Oil Red XO	73	—	—
,, ,, 101	Ponceau R	79	41	1.5
Orange No. 1	Orange 1	150	12	7.6
,, ,, 2	Orange SS	—	0	—
Yellow No. 2	Yellow AB	22	0	—
,, ,, 3	Yellow OB	61	0	—
,, ,, 5	Sunset Yellow FCF	—	17	5.0
,, ,, 4	Tartrazine	640	9	10.0

* The concentration of the enzyme solution was the same as that used for the previous experiment.

Xanthene dye				
Red No. 3	Erythrosine	773	42	1.3
,, ,, 103	Eosine	768	41	1.4
,, ,, 104	Phloxine	778	48	1.1
——	Uranine	766	0	—
Miscellaneous dye				
Green No. 2	Light Green SF yellowish	670	9	10.0
,, ,, 3	Fast Green FCF	—	8	11.1
Yellow No. 1	Naphtol Yellow S	10	0	—
Blue No. 2	Indigo Carmine	1180	0	—

FD&C: Food color No. in Japan. CI: Color Index (*7*).

1) In the concentration of 1.0 mg. dye per ml. (final).

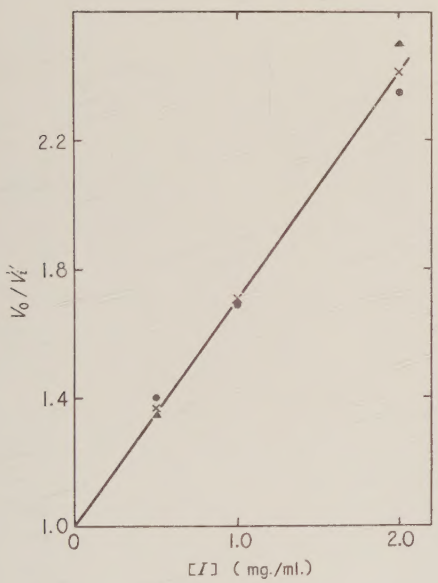

FIG. 2. Noncompetitive inhibition of pancreatic lipase by Eosine. The same conditions as shown in Fig. 1 were used.

Effect of Miscellaneous Dyes—The effects of two triphenylmethane dyes (Light Green SF yellowish and Fast Green FCF) on lipase action were tested in concentrations of 0.05, 0.1 and 0.2 per cent (w/v). These dyes had a slight effect on the lipase action, but Naphtol Yellow S and Indigo Carmine had no effect in concentrations less than 0.2 per cent (w/v) (Table I).

SUMMARY

By using a substrate, polyoxyethylene sorbitan monolaurate, which is completely soluble in water, the inhibition of pancreatic lipase by various food colors was investigated kinetically.

Water-soluble azo dyes (Ponceau 3R, Ponceau SX, Ponceau R, Orange 1 and Sunset Yellow FCF) and xanthene dyes (Erythrosine, Eosine and Phloxine) inhibited pancreatic lipase noncompetitively, while fat-soluble azo dyes (Orange SS, Yellow AB and Yellow OB) had no effect on pancreatic lipase. The two triphenylmethane dyes (Light Green SF yellowish and Fast Green FCF) had a slight effect on the lipase action noncompetitively, and Naphtol Yellow S and Indigo Carmine had no effect.

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